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INACTIVATION OF INVERTASE BY UREA[1]

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Biological Laboratories, Princeton University, New Jersey, and The Marine Biological Laboratory, Woods Hole, Massachusetts

FOUR FIGURES

INTRODUCTION

It has been shown (Osborne and Chase, '54) that the activity of *Cypridina* luciferase is inhibited by urea in low concentrations compared with those required for the denaturation of many non-catalytic proteins. It therefore seemed desirable to study other enzyme systems in this connection.

The purpose of the present paper is to report experiments with two different commercial preparations of invertase. Activity was measured at a number of urea concentrations and, as will be described, was found to vary in much the same way as reported for luciferase, except that the invertase appears to be somewhat less sensitive.

One reason for choosing invertase is the high degree of precision with which sucrose hydrolysis can be measured polarimetrically, so that reliable data are easily obtained. Also, there are several highly stable invertase preparations commercially available upon which considerable experimental work has been done.

MATERIALS AND METHODS

In the present work Difco invertase[2] was used and, as a check, representative experiments were also performed with

[1] This work was supported by a grant from the National Science Foundation and by funds of the Eugene Higgins Trust allocated to Princeton University.

[2] The Difco invertase used in this work was in liquid form received in sealed glass ampules containing 10 ml of solution. Difco Laboratories now supply this enzyme preparation in solid form to be dissolved in water before use. All statements in this paper refer to the earlier, liquid invertase preparation.

GBI (General Biochemicals, Inc.) invertase stated to be free of melibiase.[3] Neither of these preparations is probably very pure but both are highly stable.

Optical rotation measurements were made at about 26°C. with a Schmidt and Haensch polarimeter and sodium arc. Readings could be duplicated to 0.02°.

A typical experiment was conducted in the following way. Thirty milliliters of a 10% sucrose solution and a mixture of 14 ml of 0.2 *M*, pH 4.6 acetate buffer and 1 ml of enzyme solution[4] were brought separately to 26°C. in a water bath. When studying the effect of urea the solid substance was dissolved in the sucrose solution. A stopwatch was set going at the moment of mixing the sucrose and invertase solutions.

Two and one-half milliliter samples of the reaction mixture were removed at intervals and one drop of 5 *N* sodium hydroxide was added to inactivate the enzyme and cause mutarotation. The optical rotation of the samples was measured in a semi-micro polarimeter tube of 1.5 ml volume and one decimeter optical length. Ordinarily the optical rotations were all read at the end of the experiment, within three hours after making the samples alkaline.

Although the hydrolysis of sucrose by invertase does not exactly obey the equation for a first order reaction, agreement is actually good enough to permit use of the apparent first order rate constant as a measure of invertase activity. The enzyme activities in the presence of the various concentrations of urea were therefore determined in this way, and were consequently independent of the initial sucrose concentration. This method of determining the activity was particularly appropriate because it became apparent after completion of the experiments that appreciable increases

[3] Actually, this enzyme preparation was not found to be completely free of melibiase activity but it was nearly so.

[4] One milliliter of the Difco liquid preparation was used directly for most of the experiments. For the reactions run with the GBI invertase preparation an aqueous stock solution containing 6 mg/ml was first prepared, of which 1.0 ml was used for each run.

in volume occurred when large amounts of urea were added to the solutions. Consequently, the greater the amount of solid urea added, the more were the concentrations of invertase, urea, buffer and sucrose affected.

Although variable sucrose and buffer concentrations could be disregarded in analyzing the results, it was necessary to know the actual urea and relative invertase concentrations in the various experiments. These were easily determined by measuring the volumes of representative reaction mixtures when known amounts of urea had been added. These volume measurements were independently checked by determining

TABLE 1

Actual volumes and optical rotations resulting on adding to 45.0 ml of 6% sucrose solutions sufficient solid urea to have produced concentrations of 0.0, 2.0, 4.0 and 6.0 M, respectively, if there had been no increase in volume of the solution. See text for details.

UREA CONC. IF NO VOL. CHANGE HAD OCCURRED	ACTUAL RESULTING VOLUME MEASURED	ACTUAL UREA CONC. DUE TO VOL. CHANGE	OPTICAL ROTATION AT NEW VOLUME	OPTICAL ROTATION TIMES VOLUME
0.0 *M*	45.0 ml	0.0 *M*	4.01°	180.5
2.0 *M*	49.1 ml	1.8 *M*	3.69°	181.2
4.0 *M*	53.1 ml	3.4 *M*	3.42°	181.6
6.0 *M*	57.1 ml	4.7 *M*	3.16°	180.4

the optical rotation of solutions identical with experimental reaction mixtures except that water had been substituted for the invertase. Table 1, giving the results of measurements of this sort, shows the striking effect of added urea upon the volume of the solution under the present conditions. The values given for urea concentration and for relative invertase activity have, of course, been corrected throughout for this effect.

EXPERIMENTAL RESULTS

Experiments using Difco invertase were performed in duplicate in the presence of 0.3, 0.6, 1.0, 1.8, 2.6, 3.4 and 4.7 *M* urea concentrations and with no urea. As figure 1 shows, urea

decreases the rate of hydrolysis of sucrose, greater degrees of inhibition occurring the higher the urea concentration. Practically identical results were obtained with the GBI invertase (also shown in figure 1).

Although the concentration of invertase in these experiments was very low, it nevertheless seemed desirable to determine whether there was any contribution by the enzyme, alone and in the presence of urea, to the optical rotation.

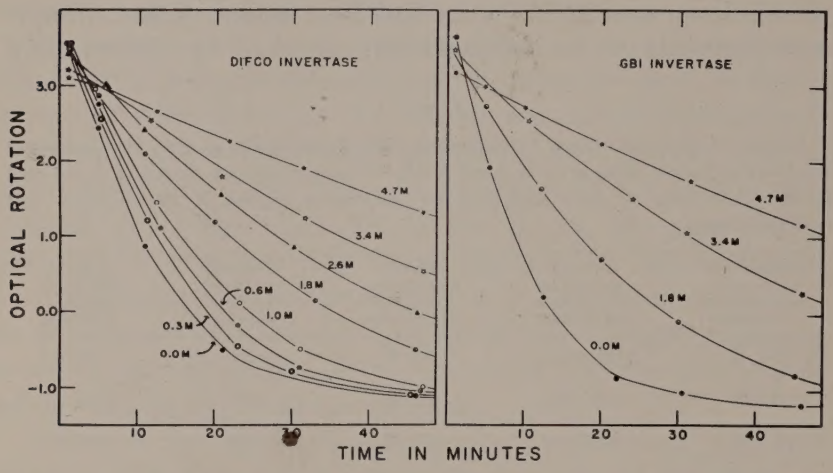

Fig. 1 Course of hydrolysis of sucrose by two different invertase preparations in the presence of various concentrations of urea, as indicated. It is evident that urea markedly inhibits the enzyme activity and that the effect is essentially the same for both preparations.

Such experiments were carried out in the usual way except that distilled water was substituted for the sucrose solution. The average polarimeter reading in the absence of urea was 0.02°, with 1.0 *M* urea it was also 0.02°, with 1.8 *M* urea it was 0.01°, and with 4.7 *M* urea it was 0.04°. No significant change occurred, furthermore, over a period of 30 minutes. Contribution of the enzyme and accompanying impurities to the optical rotation could therefore be disregarded.

It is apparent in figures 1 and 2 that the initial values of the optical rotation in the various experiments differ some-

what, being lower the higher the concentration of urea present. This is because the initial sucrose concentrations were not identical in the several experiments due to the effect, discussed above, of the added urea on the final volumes of the reaction mixtures. The derived values of invertase activity are, however, independent of initial sucrose concentration, as mentioned earlier.

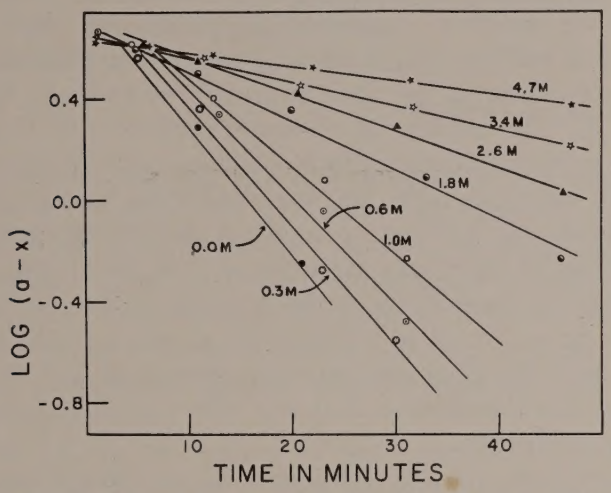

Fig. 2 The data shown in the left hand side of figure 1 plotted in terms of the equation for a first order reaction. The slopes of the straight lines drawn through the points are proportional to the activity of the enzyme. Urea concentrations are indicated.

The pH of the reaction mixture was buffered at 4.6 but in the presence of 4.7 *M* urea it rose to 5.3. Although the pH-activity curve for invertase has long been known to have a broad maximum in this pH range (Sumner and Myrbäck, '50), it nevertheless seemed desirable to determine the relative activity of the invertase at the higher pH in the absence of urea because of the possibility that under the present experimental conditions increased pH, rather than urea, might account for the observed loss of activity. Experiments run at pH 5.4 gave practically identical results with those at pH 4.6, so hydrogen ion concentration evidently plays no

role in the loss of activity of invertase at the higher urea concentrations. It does not, of course, necessarily follow that urea inactivation of invertase would be independent of pH over the entire range.

An experiment similar to that described by Chase and Brigham ('52) was carried out to determine whether or not the effect of urea on the enzyme was reversible. A buffered invertase solution, 4 *M* with respect to urea, was allowed to stand for 30 minutes. A sucrose solution was then added containing a sufficiently small amount of urea so that the urea concentration in the final mixture was reduced by dilution to 2 *M*. The change in optical rotation with time was measured in the usual way. Two other experiments were also performed identical with the one described, except that in one 2 *M* urea was present throughout and in the other the concentration of urea was maintained at 4 *M*. It was found that the rate of hydrolysis in the experiment involving dilution of the urea from 4 to 2 *M* was the same as in the experiment in which the urea concentration remained 2 *M* throughout. This result indicates that the effect of urea upon the enzyme is reversible.

Analysis of the results. Although, as mentioned earlier, it is well known that the equation for a first order reaction does not exactly describe the hydrolysis of sucrose by invertase, the fit is still sufficiently good to permit an estimation of the activity of the enzyme. The present data were, therefore, analyzed by plotting *log* $(a - x)$ against *time,* taking the polarimeter reading at any particular time, minus that when the reaction was over, as proportional to $(a - x)$. The data plotted in this form are shown in figure 2, the slopes of the straight lines being proportional to the enzyme activity. It is evident that the equation describes the experimental results quite well enough and that the enzyme activity decreases with increasing urea concentration.

Since the inactivation of invertase by urea is reversible, the same mechanism might be assumed to apply here as in the case of the inactivation by urea of *Cypridina* luciferase

(Osborne and Chase, '54). The same analysis was therefore applied, assuming a reversible combination of invertase and urea to form an inactive compound or complex involving the following equilibrium:

$$\frac{[Invertase][Urea]^n}{[Invertase\ (Urea)_n]} = K,$$

where n represents the number of urea molecules present in the inactive compound, or the number of urea molecules combining with one active catalytic site.

This equation can assume the form:

$$Log\ \frac{[Invertase]}{[Invertase\ (Urea)_n]} = -n\ Log\ [Urea] + Log\ K.$$

If, therefore, the logarithm of the ratio of the enzyme fraction remaining active to that becoming inactive at any concentration of urea be plotted against the logarithm of the urea concentration, a straight line should describe the data and the numerical value of the slope of this line should indicate the number of urea molecules reacting with one invertase molecule (or active catalytic site) to form the inactive compound or complex.

Table 2 gives the values of the relative first order rate constants for the reaction and the corresponding active enzyme fraction at the urea concentrations specified, and figure

TABLE 2

Invertase activity as a function of urea concentration. The second column gives the relative first order rate constants obtained from the lines plotted in figure 2, and the last column shows the fraction of the enzyme active at the various urea concentrations.

UREA CONCENTRATION	RELATIVE RATE CONSTANT	ACTIVE FRACTION
0.0 *M*	1.27	1.00
0.3 *M*	1.19	0.94
0.6 *M*	1.06	0.83
1.0 *M*	0.87	0.69
1.8 *M*	0.54	0.43
2.6 *M*	0.41	0.32
3.4 *M*	0.27	0.21
4.7 *M*	0.18	0.14

3 shows the plot of *log (active fraction/inactive fraction)* vs. *log (urea concentration)*. A straight line describes the data very well and the slope of the line has a value of 1.7, which may be interpreted as meaning that 1.7 urea molecules combine reversibly with each invertase molecule (or active catalytic site) to cause inactivation.

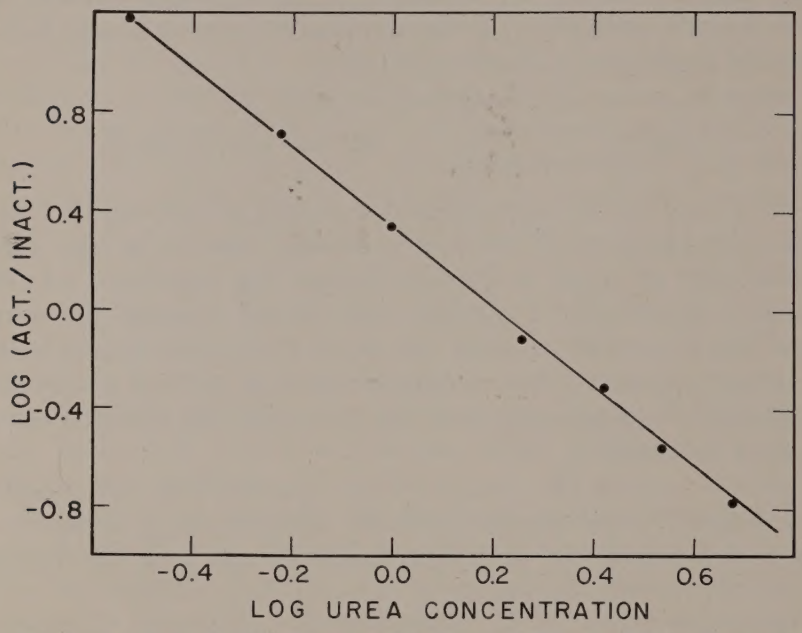

Fig. 3 The data of table 2 presented in terms of the equation for a reversible combination of enzyme and urea to give an inactive compound. The numerical value of the slope of the line, 1.7, represents the number of urea molecules combining with one enzyme molecule, or active catalytic site, to cause inactivation. See text for details.

DISCUSSION AND CONCLUSIONS

The results from these experiments on the inactivation of invertase by urea are qualitatively similar to those of Osborne and Chase ('54) with *Cypridina* luciferase. However, as shown in figure 4, invertase would appear to be the less sensitive of these two enzymes, unless the difference is due to impurities with which the urea can combine. In the case

of luciferase a 0.6 *M* urea concentration caused 50% inhibition, whereas a 1.5 *M* concentration of urea was required to inactivate invertase to the same extent. Even so, invertase would appear to be inhibited by considerably lower urea concentrations than those required to affect such properties as the optical rotation and viscosity of certain purified proteins (Kauzmann, et al., '53).

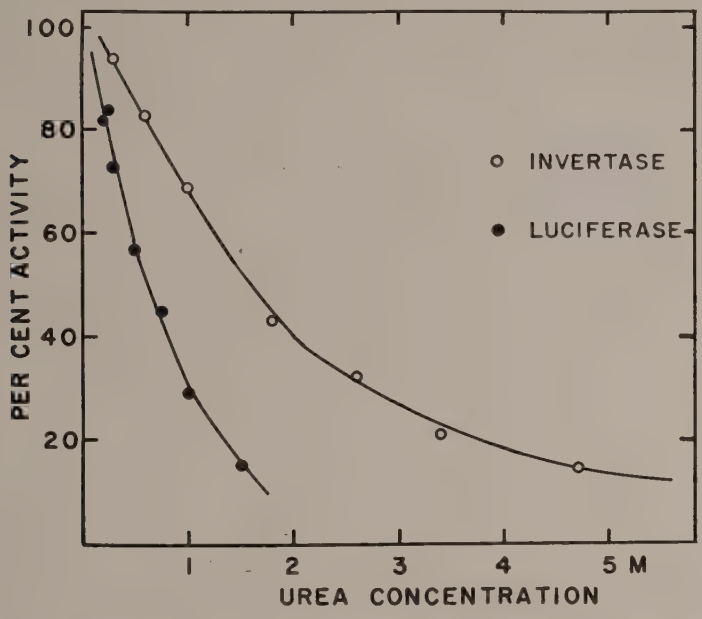

Fig. 4 A comparison of the inactivating effect of urea upon invertase with its effect upon *Cypridina* luciferase (Osborne and Chase, '54). Luciferase is evidently about two and a half times as sensitive to urea, unless impurities are affecting the results.

Possibly the difference in the sensitivities of invertase and luciferase toward urea may be related to their very different functions, invertase catalyzing an hydrolysis and luciferase probably belonging with the oxidative enzymes (Harvey, '52). A more extensive study of the effect of urea on the activities of hydrolytic and oxidative enzymes might yield information on this point. It is interesting that in the case of both

luciferase and invertase inactivation appears to involve the reversible combination of one enzyme molecule (or active catalytic site) and about 1.7 urea molecules, although experiments comparing a number of different enzymes would be necessary before any general significance could be attached to this ratio.

In the case of luciferase, Osborne and Chase ('54) reported two distinct effects of urea; one instantaneous and the other relatively slow. Kauzmann and Simpson ('53) have shown that bovine plasma albumin, β-lactoglobulin, ovalbumin and pepsin behave quite differently with respect to the effect of urea upon their optical rotation. That of solutions of the albumin undergoes an instantaneous increase with no further change on continued exposure to urea, whereas the optical rotation of lactoglobulin solutions shows an instantaneous change followed by a slower one very dependent upon urea concentration and pH. In the case of the ovalbumin, only a slow change in optical rotation occurs, whereas pepsin solutions show no effect of even high urea concentrations.

So far, no evidence exists for any other than an immediate reversible inactivation of invertase by urea. One experiment was performed in which the invertase was allowed to stand in the presence of 4 *M* urea for two hours before sucrose was added and the rate of hydrolysis determined. At the same time a similar experiment was carried out in which the rate of hydrolysis was measured immediately after mixing the urea, sucrose and enzyme. The velocity constants of these two reactions were the same, indicating that there was no further inhibition of the invertase on prolonged exposure to urea.

As in the case of the inactivation of *Cypridina* luciferase by urea, little more than conjecture can be offered in the way of an explanation for the effect of urea upon invertase. Since the reaction is easily reversible it is difficult to imagine any very major change occurring in the structure of the molecule. This conclusion would be further supported by the fact that the data indicate only one or two urea molecules as combining

with the enzyme to form the inactive complex. Were an extended unfolding involved, many more urea molecules would presumably participate.

Further experiments are contemplated on the effect of urea upon invertase under other conditions of pH and temperature. It is quite conceivable that if such a change as partial unfolding of the molecule accompanies heat treatment, for example, this might be intensified in the presence of urea with a resultant increase in the degree of inactivation of the enzyme, and possibly with the appearance of an additional, irreversible step or steps in the inactivation process.

SUMMARY

Two preparations of yeast invertase were studied with respect to their inactivation by urea at pH 4.6 and 26°C. Both behaved identically.

Enzyme activity was determined by measuring the hydrolysis of sucrose polarimetrically and applying the equation for a first order reaction to the data. The rate constant obtained was taken as proportional to enzyme activity.

As in the case of *Cypridina* luciferase (Osborne and Chase, '54), an immediate, reversible inactivation of invertase occurred in the presence of urea. The invertase was apparently less sensitive than luciferase, 0.6 *M* urea causing 50% inactivation of the latter whereas 1.5 *M* urea was required to inactivate invertase to the same extent. In the case of invertase, as also in the previous studies on luciferase, a simple mass law formulation showed that about 1.7 urea molecules were required per enzyme molecule, or active catalytic site, to form the hypothetical inactive complex. There was no evidence that any inactivation of invertase other than a rapid, reversible one occurred at the pH and temperature of these experiments.

Invertase, like luciferase, seems to be inactivated by lower concentrations of urea than ordinarily required to affect such properties of proteins as optical rotation and viscosity.

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DISTRIBUTION OF THE TRICARBOXYLIC ACID CYCLE ENZYMES IN EXTRACTS OF ESCHERICHIA COLI

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Department of Bacteriology, Communicable Diseases Division, Walter Reed Army Institute of Research, Washington, D. C.

SEVEN FIGURES

It is now certain that the major pathway of terminal respiration in aerobically grown *Escherichia coli* proceeds *via* the citric acid cycle (Swim and Krampitz, '54; Wheat and Ajl, '54; Ajl and Wong, '55). All of the individual oxidative steps, however, have not yet been well characterized. In order to elucidate the mechanism of the component enzymatic reactions of the tricarboxylic acid cycle in this bacterium the separation and purification of the individual enzymes was clearly indicated. An attempt in this direction has been made and the results obtained are described in this report.

The enzymes of the Krebs cycle have been partially separated, their cofactor requirements studied and their relative abundance in cell-free extracts estimated. This work was made possible by the findings of Swim and Krampitz ('52) and Wheat and Ajl ('54) that all substrates participating in the Krebs cycle can be oxidized by cell-free extracts.

METHODS

The organism employed in this work was *Escherichia coli*, strain E26. It was grown for 18 hours at 37°C. with vigorous aeration in an acetate medium (Ajl, '50) containing 0.2% yeast extract.

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Preparation of cell-free extracts for fractionation. Cell-free extracts were prepared by alumina grinding (McIlwain, '48) in the following manner. A large mortar, previously chilled to — 20°C., was evenly lined with a known quantity of cells. This procedure immediately freezes the bacteria against the walls of the vessel. An equal amount of alumina 303 powder was then added and mixed with the frozen cells by scraping the latter off the sides of the mortar. The mixture of powder and cells was triturated until a rather thick, creamy mass was obtained. The cell paste was diluted (with trituration) four-fold with either distilled water or dilute phosphate buffer (pH 7.0), allowed to stand 5 to 10 minutes and centrifuged at low speed to remove cell debris and alumina powder. To obtain translucent extracts, the supernate was centrifuged twice at approximately 12,000 rpm. Protein values ranged from 20 to 30 mg per ml. The supernatant extracts obtained by this procedure are rather viscous making subsequent ammonium sulfate fractionations extremely difficult. It has been found in this laboratory (Wheat and Ajl, '55) and by Stanier and Ingraham ('54) that this difficulty can be obviated by the treatment of the extracts with small amounts of DNA'se [2] in the following manner. Approximately 500 μg of DNA'se powder are stirred into 100 ml of extract previously adjusted to 10^{-3} M with respect to Mg ions. The extract is incubated at 0°C. until the viscosity is decreased to a minimum as determined by the rate of flow from a 0.2 serological pipette. The extract thus obtained is now fractionated at 0° to 3°C. by slowly adding the requisite amounts of solid ammonium sulfate [3] with constant stirring. The protein precipitate obtained at the desired salt concentration is centrifuged off, redissolved in 0.5 M phosphate buffer, pH 7.0, centrifuged again to remove any insoluble material and kept frozen prior to use.

[2] The following abbreviations are used: desoxyribonuclease, DNA'se; triphosphopyridine nucleotide, TPN; diphosphopyridine nucleotide, DPN; coenzyme A, CoA; tris (hydroxymethyl) aminomethane, Tris.

[3] Per cent ammonium sulfate saturation was calculated directly by assuming 70 gm of the salt per 100 ml of solution at 0°C. to be 100% saturated.

Spectrophotometric measurements of enzymatic activity. The enzymatic activities of aconitase and isocitric, *alpha*-ketoglutaric, malic, lactic and glutamic dehydrogenases were followed in the Beckman DU spectrophotometer in the presence of either TPN or DPN and the requisite substrates. Fumarase activity was detected by an increase in absorption at 240 mμ with malate as substrate (Racker, '50). The color of the formazan derivative produced as a result of the reduction of 2, 3, 5-triphenyl tetrazolium chloride by succinic acid in the presence of enzyme was used as a qualitative index of succinic dehydrogenase activity (Glock and Jensen, '53). Acetate activating enzyme and condensing enzyme activities were measured according to the methods of Novelli and Lipmann ('50). Oxalacetate decarboxylase activity was determined manometrically and was based on the rate of decarboxylation of oxalacetic acid in the presence of enzyme, substrate, Mn ions and buffer (Mehler et al., '48). Succinoxidase activity was likewise measured in the usual Warburg apparatus where O_2-uptake was used as an index of its activity.

Protein determination. Protein concentrations were estimated by the turbidimetric method of Stadtman et al. ('51).

EXPERIMENTAL

Distribution of tricarboxylic acid cycle enzymes in extracts of E. coli. The overall distribution of a number of tricarboxylic acid cycle enzymes including glutamic dehydrogenase in cell-free extracts of *E. coli* is shown in figure 1. The concentration of the various enzymes in the individual ammonium sulfate fractions was estimated on the basis of enzyme activity. Where enzymatic activity could be determined spectrophotometrically by reduction or oxidation of either DPN or TPN as in the case of aconitase and isocitric, *alpha*-ketoglutaric, glutamic and malic dehydrogenases, a unit of activity was defined as the change in log Io/I of 0.01 per minute. The increment in optical density, *i.e.*, log Io/I, between the reading at 15 and 45 seconds after mixing of the solution, multiplied by 2, was taken as the enzymatic activity per minute. Fuma-

rase activity was calculated using Racker's ('50) method where an enzyme unit is defined as the amount of enzyme causing an increase in optical density of 0.001 per minute at 240 mμ. Acetate activating enzyme and condensing enzyme activities in the various fractions were estimated in terms of micromoles of hydroxamic acid and citric acid formation, respectively. The quantity of oxalacetate decarboxylase was

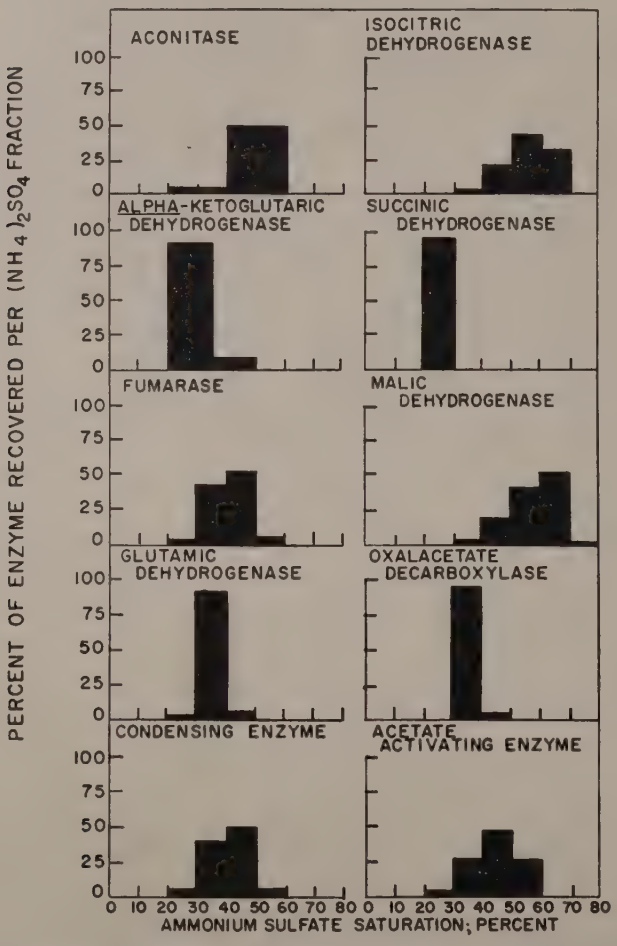

Fig. 1 Distribution of tricarboxylic acid cycle enzymes in crude extracts of *Escherichia coli*. Initial pH of extract 6.8.

determined in terms of CO_2 production. All of the above mentioned determinations were performed with known aliquots of each of the ammonium sulfate fractions shown in figure 1. The total activity of each fraction was obtained by multiplying the activity per milliliter by the volume of enzyme. The activities of the individual fractions were added and assumed to represent 100% of enzyme present. The units of enzyme activity in each fraction divided by the total units contained in all fractions $\times$ 100 represented the recovery of enzyme in per cent in each fraction.

The separations depicted in figure 1 were effected only by ammonium sulfate fractionations. The shaded areas represent the relative concentrations of each of the enzymes in the various ammonium sulfate fractions and do not necessarily reflect either their specific activities or their relative abundance in total unfractionated extracts or whole cells. Generally speaking, *alpha*-ketoglutaric, succinic and glutamic dehydrogenases and fumarase are associated with protein fractions precipitable below 50% saturation whereas isocitric and malic dehydrogenases can be salted out from solution at ammonium sulfate concentrations considerably above 50%. The acetate activating and condensing enzyme systems can be precipitated out at approximately 60% ammonium sulfate saturation.

In order to determine the relative concentration of each of the enzymes of the tricarboxylic acid cycle in crude extracts as well as in individual ammonium sulfate fractions, their specific activities were determined. Specific activity of enzyme is expressed as micromoles per minute per milligram protein. In the case of those enzymes whose activities are measured in terms of reduction or oxidation of the pyridine nucleotides, the specific activity can be calculated in the following manner. The molecular extinction coefficient, β, for reduced TPN or DPN at 340 mμ is 6.22×10^6 cm^2 per mole (Horecker and Kornberg, '48). Since $\beta = \log Io/I/_{c \times d}$, a change in optical density (log Io/I) of 0.01 in a 1 cm cuvette will correspond to a concentration, c, of either TPN red or DPN red of $c = 0.01/6.22 \times 10^6$ cm$^2 \times 1$ cm $\times$ mole^{-1} or c

$= 0.161 \times 10^{-8}$ moles/ml, and for a volume of 3 ml 0.483×10^{-8} moles or 0.00483 μM per 3 ml per optical density change of 0.01. Assuming that the reaction among enzyme, substrate and TPN or DPN is stoichiometric, it is possible to calculate the specific activity, *i.e.*, μM/min/mg protein by multiplying 0.00483 by the number of enzyme units (previously defined) per milligram protein. In the case of fumarase, the same calculations were applied using the conversion factor given by Ochoa ('51) where one enzyme unit is equivalent to 0.00474×10^{-7} moles of l-malic acid dehydrated per milliliter per minute or 0.01422 μM per 3 ml per minute. The specific activities of acetate activating and condensing enzymes are expressed as micromoles of hydroxamic acid and citric acid formation respectively per minute per milligram of enzyme. Micromoles of CO_2 produced per minute per milligram protein was used to determine the specific activity of oxalacetate decarboxylase.

The specific activities as well as the total concentrations of a number of tricarboxylic acid cycle enzymes in crude extracts as well as in various ammonium sulfate fractions are given in table 1. Assuming that the methodology employed for the determination of each of the enzymes is equally sensitive it becomes possible to compare their relative concentrations in crude extracts. It would appear that acetate activating and condensing enzyme systems and aconitase are present in relatively small amounts whereas the concentrations of fumarase, isocitric, and malic dehydrogenases and oxalacetate decarboxylase are considerable. The total concentrations of these enzymes recovered after ammonium sulfate treatment follows essentially the same pattern. It is to be noted, however, that the per cent recovery of each of the enzymes varies from approximately 20 to 500%. The low recoveries are undoubtedly due to the partial inactivation of the enzymes whereas the apparent recoveries above the theorectical 100% reflect the removal of interfering substances by ammonium sulfate treatment.

By comparing the specific activities of the various enzymes in crude extracts with the specific activities of the same

TABLE 1

Relative concentration of tricarboxylic acid cycle enzymes in crude and ammonium sulfate fractionated extracts of Escherichia coli

$(NH_4)_2SO_4$ FRACTION	PROTEIN CONC. mg [1]	SPECIFIC ACTIVITY OF ENZYMES; μM/MIN/MG PROTEIN						
		Acetate activating system	Condensing enzyme system	Aconitase	Isocitric dehydrogenase	Fumarase	Malic dehydrogenase	Oxalacetate decarboxylase
0.0–0.2	1.2	0.000	0.000	0.000	0.610	0.000	2.460	0.000
0.2–0.3	25.0	0.008	0.020	0.000	0.096	0.091	0.070	0.000
0.3–0.4	105.0	0.034	0.033	0.000	0.074	0.584	0.980	0.420
0.4–0.5	254.0	0.024	0.018	0.006	0.137	0.658	0.680	0.086
0.5–0.6	52.0	0.066	0.096	0.029	0.200	0.353	7.950	0.000
0.6–0.7	34.0	0.000	0.000	0.000	4.800	0.042	25.500	0.000
0.7–0.8	15.0	0.000	0.000	0.000	0.182	0.000	2.550	0.000
Crude extract	783.0	0.024	0.056	0.016	0.364	0.310	0.418	0.120
Enzyme recovery in all fractions; total units [2]		13.5	13.5	3.0	316.0	251.0	1615.0	66.0
Enzyme conc. in crude extract; total units [3]		18.8	43.8	12.5	285.0	243.0	327.0	94.0
Apparent recovery of enzymes in all fractions; %		72.0	31.0	24.0	111.0	103.0	494.0	70.0

[1] Total protein recovery of combined ammonium sulfate fractions was 486 mg or 62% of the starting material.

[2] Total units of enzyme recovery in all fractions equals the sum that is obtained by multiplying the specific activities by the milligrams of protein of each of the ammonium sulfate fractions.

[3] Total units of enzyme in crude extracts equals the specific activity multiplied by the total amount of protein.

enzymes in certain of the ammonium sulfate fractions it becomes evident that a certain degree of purification has been achieved. The range of purification varied from two to sixty fold. For example, the specific activity of the acetate activating system in the crude extract was found to be 0.024 μM/min/mg protein. The same enzyme system when precipitated between 0.5 and 0.6 ammonium sulfate saturation had a specific activity of 0.066 μM/min/mg protein; representing a three fold purification. Comparing the specific activity of malate in the crude extract and in the 0.6 to 0.7 ammonium sulfate fraction, a sixty fold purification was obtained. It is to be noted that the most active protein fraction is usually one containing the bulk of the enzyme. This becomes evident when the data presented in table 1 and figure 1 are compared. For example, the data in figure 1 reveal that the ammonium sulfate fraction between 0.6 and 0.7 saturation contains the bulk of malic dehydrogenase. The data in table 1 reveal that the highest specific activity of this enzyme is likewise found in this fraction. Furthermore, it is interesting to note that certain of the enzymes of the tricarboxylic acid cycle are concentrated in the same ammonium sulfate fractions (see fig. 1). This is true of (a) the acetate activating and condensing enzymes, (b) malic and isocitric dehydrogenases, and fumarase and aconitase.

Studies of individual enzymes

Aconitase. Aconitase, the enzyme responsible for the conversion of citrate to an equilibrium mixture of *cis*-aconitate and isocitrate is precipitable from crude *E. coli* extracts between 40 and 60% ammonium sulfate saturation. The test for the enzyme is based upon the formation of isocitric acid from either citric or *cis*-aconitic acids in the presence of aconitase, and its reaction with TPN when both Mg ions and an excess of isocitric dehydrogenase-oxalosuccinic carboxylase are available. The reduction of TPN under these conditions becomes a measure of aconitase activity. In figure 2 are shown

the relative rates of TPN reduction by crude extracts with either citrate, *cis*-aconitate or isocitrate as substrate. From the data presented, it would appear that the rate of conversion of citrate to isocitrate or *cis*-aconitate is considerably

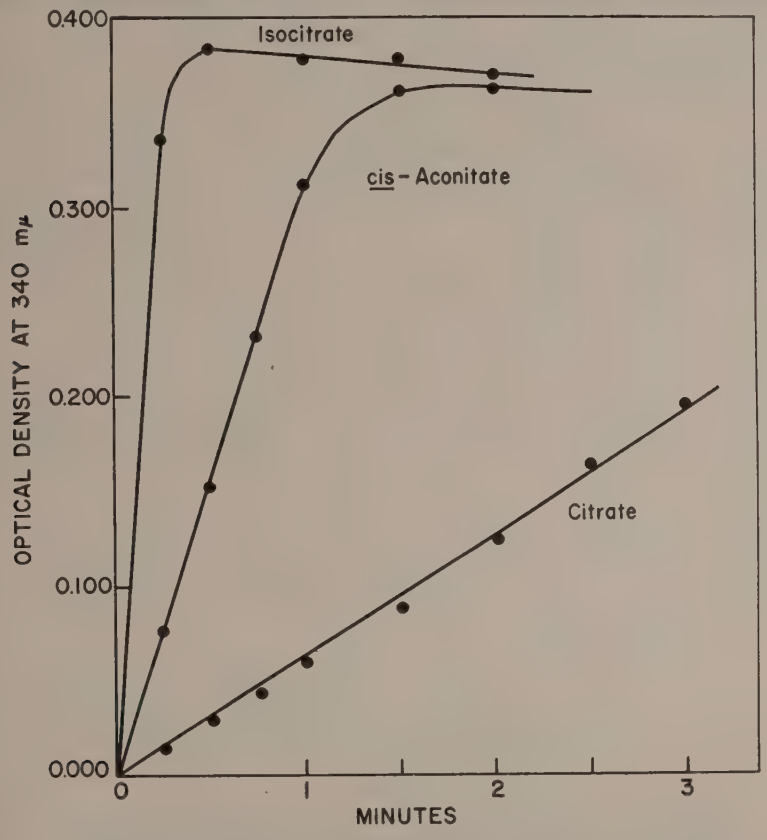

Fig. 2. Optical measurements of isocitric dehydrogenase and aconitase in crude extracts of *Escherichia coli*. Total volume of reactants 3.0 ml. Each cuvette contained 2.3 mg of protein, 0.2 mg of TPN, 100 μM of phosphate buffer, pH 7.4, 0.5 μM $MgCl_2$ and 5 μM of either citrate, *cis*-aconitate or isocitrate. Readings were taken 15 seconds after addition of substrate to experimental cuvette.

lower than the reduction of TPN in the presence of isocitric acid. Consequently, aconitase must be thought to constitute the limiting factor of TPN reduction in the presence of citrate or *cis*-aconitate.

Partial purification of aconitase was achieved by ammonium sulfate fractionation. It is evident from the data presented in figure 3 that the rate of TPN reduction is approximately doubled when equivalent amounts of purified aconitase are substituted for crude extracts. All of the experiments de-

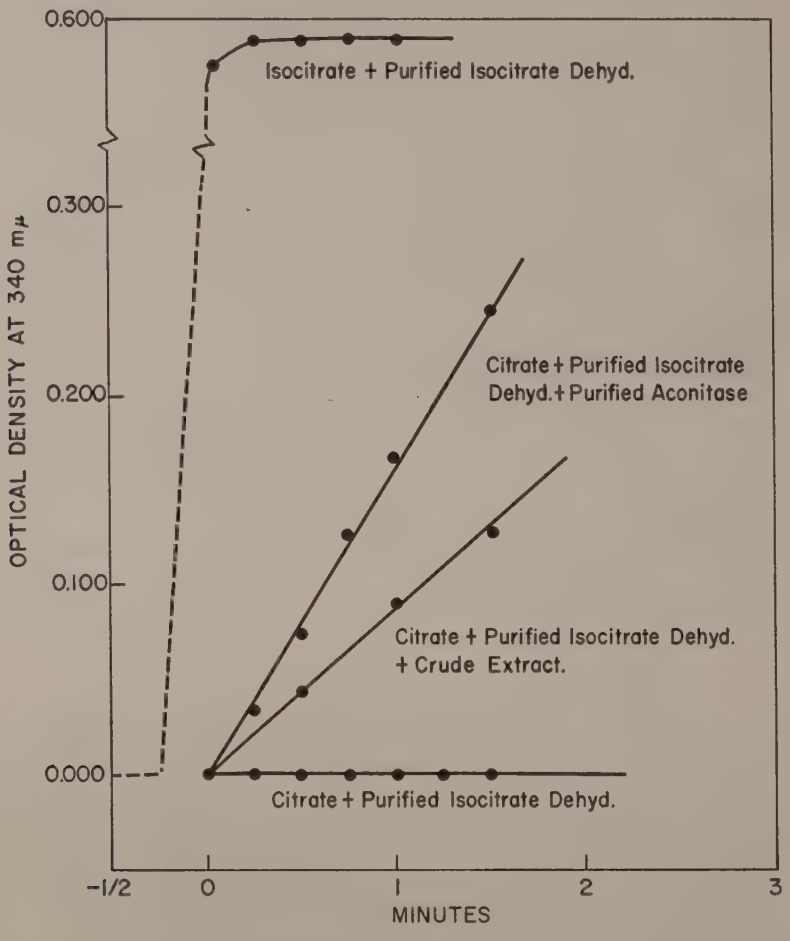

Fig. 3 Test system for aconitase purification. Each cuvette contained 5 μM of substrate, 100 μM of phosphate buffer, pH 7.4, 5 μM of $MgCl_2$, 0.4 mg of TPN and excess isocitric dehydrogenase. Where indicated 2.6 mg of either crude extract or purified aconitase were added. Readings were taken 15 seconds after addition of substrate to experimental cuvette. Total volume 3 ml.

scribed in figure 3 were performed with an excess of purified isocitric dehydrogenase which was free of aconitase. An excess of the former was added so that the reduction of TPN in the presence of citrate would be limited by the amount of aconitase added. The purified aconitase represents an ammonium sulfate fraction obtained at 0.5 to 0.6 saturation. From the data presented in table 1, it is also apparent that the specific activity of the aconitase doubled when it was separated from the crude extract by ammonium sulfate precipitation at 0.5 to 0.6 saturation. Further attempts to purify aconitase failed since, as it is well known, this enzyme is extremely labile and difficult to handle. It is labile at low and high pH and is inactivated by dialysis (Wheat and Ajl, '54). Preliminary attempts to reactivate the enzyme with Fe ions in the presence of cysteine as Dickman and Cloutier ('50) have done for aconitase from animal tissues did not yield satisfactory results. Barban ('52) however, did succeed in partially purifying this enzyme from *E. freundii* by alcohol precipitation.

Isocitric dehydrogenase. Although the enzyme isocitric dehydrogenase has been shown to occur in a number of bacteria, *i.e.*, *E. coli* (Barban, '53; Swim and Krampitz, '52; Wheat and Ajl, '54), *Propionibacterium pentosaceum* (Delwiche and Carson, '52), *Corynebacterium creatinovorans* (Fukui and Vandemark, '52), *Pseudomonas fluorescens* (Barret and Kallio, '53) its characteristics have been investigated only in the case of *E. freundii* by Barban and Ajl ('52). In the present work, isocitric dehydrogenase has been purified several fold by treatment with protamine sulfate and calcium phosphate gel combined with ammonium sulfate fractionation. The procedures employed as well as the extent of purification is shown in table 2. A typical experiment consisted of dialyzing the ammonium sulfate fractions rich in isocitrate dehydrogenase (usually between 0.4 and 0.7 saturation) and treating the latter with small amounts of protamine sulfate. Protamine sulfate was used in the following manner. To an enzyme solution containing 2100 mg of protein in a volume of 70 ml were added

120 mg of protamine sulfate. The precipitate was centrifuged off and to the supernate again added 80 mg of the protamine. After a second centrifugation, approximately half of the original protein remained in the supernate together with the bulk of the enzyme. Five hundred and twenty milligram of freshly prepared calcium phosphate gel (Kunitz, '52) were added, and after 20 minutes of standing, the precipitate containing very little enzyme was centrifuged off and discarded. This procedure was repeated. The final supernate, containing the partially purified enzyme, was concentrated by ammonium sulfate precipitation. As is evident from the data in table 2,

TABLE 2

Partial purification of isocitric dehydrogenase from Escherichia coli

PURIFICATION STEP	TOTAL UNITS	SPECIFIC ACTIVITY
	μM/min/total protein	*μM/min/mg protein*
Dialyzed $(NH_4)_2SO_4$ fraction	1470	0.700
Protamine sulfate	1485	1.250
Calcium phosphate gel	1600	2.010

this protocol might be used for further purification studies since little or no enzyme is lost during the various fractionation procedures.

Alpha-ketoglutaric dehydrogenase. A DPN dependent *alpha*-ketoglutaric dehydrogenase was found to be associated with ammonium sulfate fractions precipitable below 60% saturation. Large amounts of CoA were required before DPN reduction ensued and TPN did not function. Typical results are shown in figure 4. No attempt was made to demonstrate deacylase activity. CoA dependence of DPN reduction by *alpha*-ketoglutaric dehydrogenase of bacterial origin has been demonstrated by Lindstrom ('53). *Alpha*-ketoglutaric dehydrogenase from *E. coli* appears to be refractory to pH changes. It was equally active when precipitated from solution at either pH 5.4 or 7.8.

Succinic dehydrogenase and oxidase. Succinic dehydrogenase was precipitated from crude *E. coli* extracts below 30% saturation (see fig. 1). The reduction of tetrazolium salts in the presence of succinic acid and enzyme was used as a measure of succinic dehydrogenase activity. Dialysis inactivated the enzyme. Activity could be restored on the addition

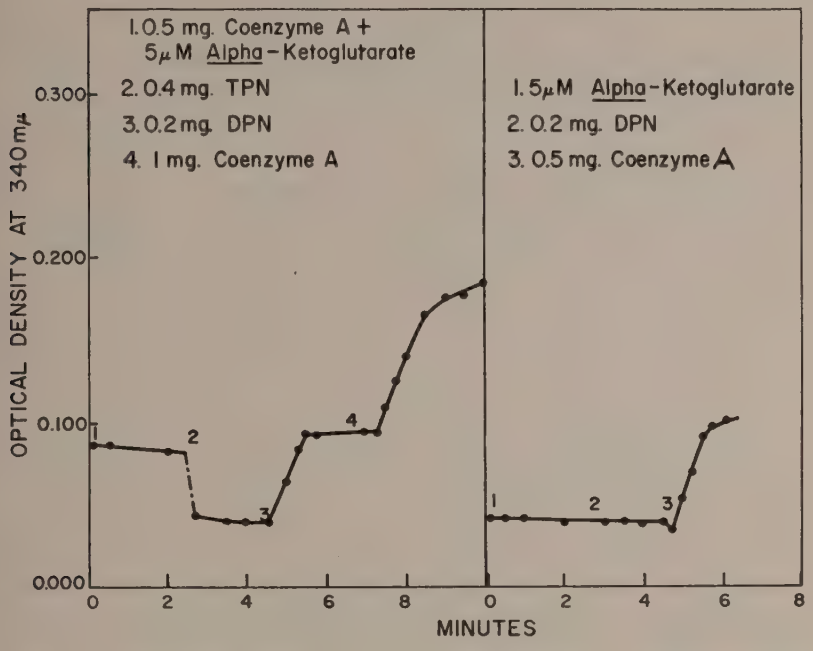

Fig. 4 Relationship between CoA and DPN reduction by *alpha*-ketoglutaric dehydrogenase. Each cuvette contained 100 μM of K_2HPO_4 and 0.31 mg of protein (ammonium sulfate fraction between 0.2 and 0.35 saturation). Additions of substrate and cofactors were made as indicated. CoA was added after it was incubated for two minutes with 50 μM of freshly neutralized cysteine.

of either a supernate of boiled cells or phosphate buffer. Thus, the original observation by Hersey and Ajl ('51) that phosphate is required for succinic dehydrogenase activity was confirmed (table 3).

Succinoxidase activity appears to be at least partially associated with particulate matter which can be brought down, in

part, by high speed centrifugation. In table 4 are shown a series of O_2-uptake data on a number of tricarboxylic acid cycle substrates with a crude cell-free extract preparation before and after high speed centrifugation. It is readily apparent that the only substrate that was oxidized by the precipitate remaining after 30 minutes of centrifugation at 20,000 rpm was succinic acid.

TABLE 3

Reductive behavior of succinic dehydrogenase from Escherichia coli in tetrazolium salts in the presence of succinic acid

EXP. NO.	ENZYME PREPARATION PLUS TRIS BUFFER	ADDITIONS	REDUCTION TIME OF DYE	
			Before dialysis *min.*	After dialysis *min.*
1	Crude extract	Succinate	60	infinite
		Succinate and phosphate	30	90
		No succinate or phosphate	180	infinite
		Phosphate without succinate	180	infinite
2	Crude extract	Succinate plus phosphate	3	..
	0.2–0.3 $(NH_4)_2SO_4$ sat.		10	..
	0.3–0.4 $(NH_4)_2SO_4$ sat.		60	..
	0.4–0.5 $(NH_4)_2SO_4$ sat.		infinite	..
3	Crude extract	Succinate	10	infinite
		Succinate plus supernate of boiled suspension of *E. coli*	..	90

Where indicated the following additions were made: 5 μM of succinate, 100 μM of phosphate buffer, pH 7.4, 5 μM of 2,3,5-triphenyltetrazolium chloride neutralized to pH 7.4 and 0.4 ml of enzyme. Total volume 1 ml. Room temperature. Dialysis was carried out overnight at 5°C. against 100 volumes of distilled water.

Malic dehydrogenase. From the specific activity data presented in table 1 it would appear that malic dehydrogenase is one of the most active enzymes of the tricarboxylic acid cycle. Ammonium sulfate fractionation resulted in a sixty fold purification. Gale and Stephenson ('39) and Booth and Green ('38) reported that this enzyme is DPN dependent. The data in figure 5, however, reveal that both DPN and TPN function

although DPN is much more effective. The test system depended upon the oxidation of reduced TPN or DPN in the presence of either crude or partially purified malic dehydrogenase with oxalacetate as substrate. The ammonium sulfate fraction between 0.6 and 0.7 saturation was free of lactic dehydrogenase since reduced TPN or DPN was not oxidized in the presence of pyruvate.

TABLE 4

Association of succinoxidase activity with particulate matter obtained from cell-free extracts of Escherichia coli

FRACTION	μL O_2-UPTAKE ON					
	Acetate	Citrate	*Alpha* keto-glutarate	Malate	Succinate	Endog.
Crude extract	118	308	328	248	364	87
Precipitate after high speed centrifugation	0	6	9	0	142	0
Supernate after high speed centrifugation	0	294	292	265	386	88

Total volume of reactants 2.3 ml. Each vessel contained 25 μM of substrate (except endogenous), 100 μM of phosphate buffer, pH 7.0, 0.3 ml of NaOH in center well and 1 ml of enzyme from a total volume of 20 ml (in the case of crude extract and supernate after high-speed centrifugation). The total precipitate obtained after 30 minutes of centrifugation of 20 ml of extract at 29,000 rpm in the Spinco ultracentrifuge was taken up in 6 ml of distilled water and 1 ml of the opalescent solution used per vessel.

Fumarase. Fumarase fulfills an essential role as a link in the chain of enzymes that catalyze the oxidation of carbohydrates, fatty acids, and some amino acids *via* the tricarboxylic acid cycle. The actvity of this enzyme can be measured by the rate of change in optical density at 240 mμ with l-malate as substrate. A typical experiment is shown in figure 6. The enzyme solution used in this experiment represented a preparation which had been treated with $MnCl_2$, absorbed and eluted from a calcium phosphate gel and concentrated by ammonium sulfate precipitation between 0.3 and 0.5 saturation. The en-

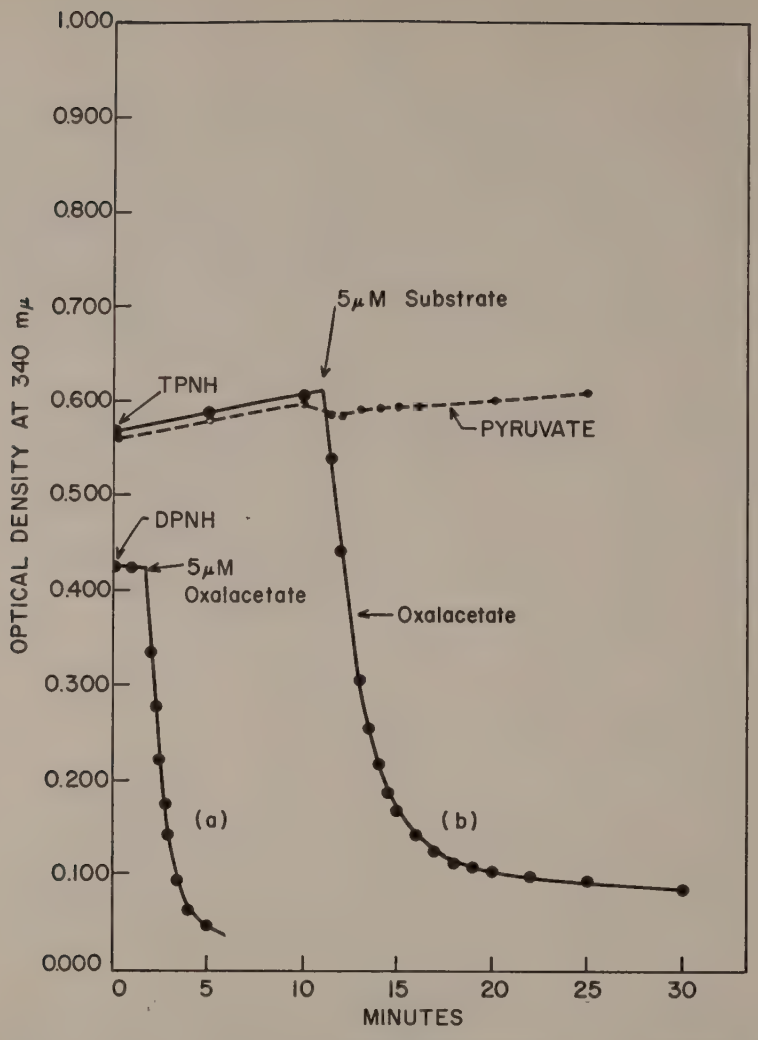

Fig. 5 Coenzyme specificity of malic dehydrogenase from *Escherichia coli*. Conditions: (a) 100 μM of phosphate buffer, pH 7.4, 5 μM of oxalacetate, enough reduced DPN to give a reading of 0.400 to 0.600 optical density and 1.25 mg protein.* (b) 100 μM of phosphate buffer, pH 7.4, 5 μM of either oxalacetate or pyruvate, enough reduced TPN to give a reading between 0.400 and 0.600 optical density and 500 μg of protein.*

Reduced TPN was obtained by the glucose-6-PO_4 dehydrogenase system in the following manner. Glucose-6-PO_4 (5 μM) was incubated in the presence of 1 mg of Zwischenferment, 100 μM phosphate buffer, pH 7.4, and 5 μM of Mg ions and 2 mg of oxidized TPN. Reaction was allowed to proceed almost to completion at which time the mixture was boiled (to destroy the excess Zwischenferment), centrifuged and an aliquot of the supernate added to the malic dehydrogenase test system.

* Ammonium sulfate fraction obtained at 0.6 and 0.7 saturation.

zyme thus obtained was relatively stable to dialysis, freezing and thawing and pH changes.

Oxalacetate decarboxylase. When crude cell-free extracts of *E. coli* were adjusted to a pH of 6.8, treated with DNA'se

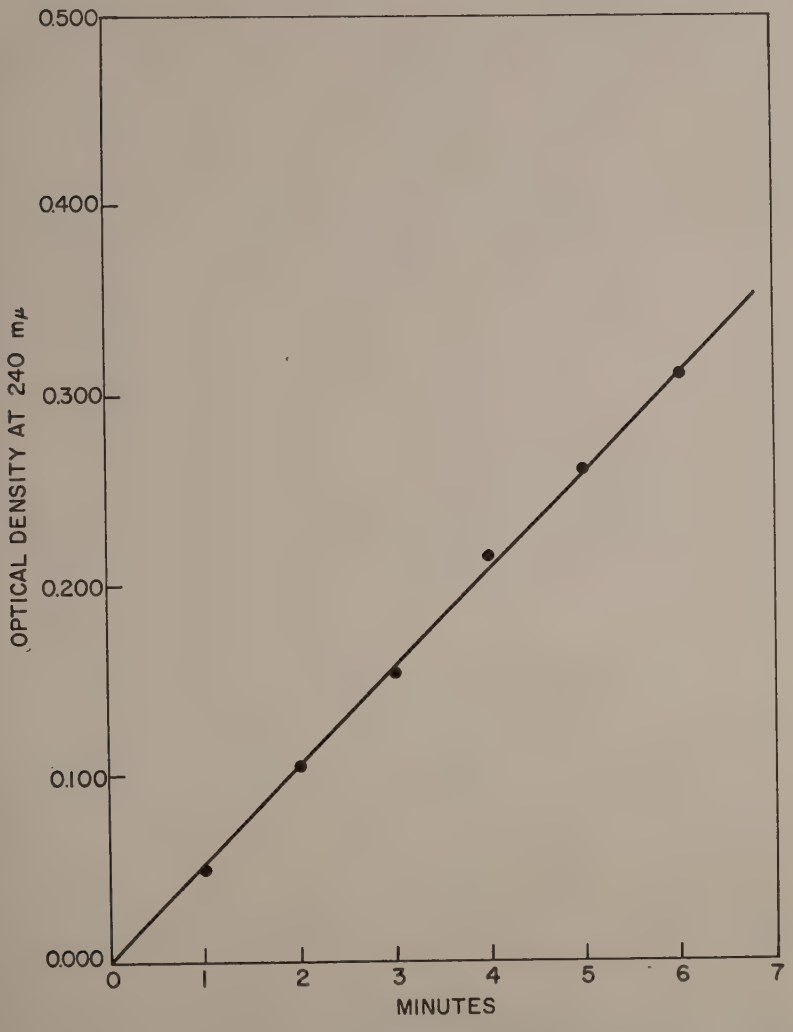

Fig. 6 Racker's optical test for fumarase. Each cuvette contained 100 μM of phosphate buffer, pH 7.4 and 93 μg of protein. Readings were started 15 seconds after addition of 10 μM of l-malate to experimental cuvette. Total volume 3 ml.

and fractionated with solid ammonium sulfate at 0°–3°C., the bulk of oxalacetate decarboxylase was precipitated between 0.3 and 0.5 ammonium sulfate saturation (table 5). It is likely that under different experimental conditions this enzyme is precipitable from solution at different levels of ammonium sulfate saturation since Kalnitsky and Werkman ('44) reported the bulk of oxalacetate decarboxylase to be associated with protein fractions above 45% ammonium sulfate saturation.

TABLE 5

Oxalacetate decarboxylase activity in fractionated extracts of Escherichia coli

$(NH_4)_2SO_4$ FRACTIONS	PROTEIN CONC. PER VESSEL	CO_2 EVOLVED	SPECIFIC ACTIVITY
	mg	*μl* *	*μM/min/mg protein*
0.0–0.2	0.02	0	0.000
0.2–0.3	0.25	0	0.000
0.3–0.4	1.00	50	0.420
0.4–0.5	2.50	25	0.086
0.5–0.6	0.50	1	0.020
0.6–0.7	0.30	0	0.000

Each Warburg vessel contained 200 μM of acetate buffer, pH 5.4, 5 μM of Mn ions, 25 μM of sodium oxalacetate and the indicated amounts of enzyme. The figures shown in table are corrected for spontaneous decarboxylation of oxalacetate. Time of incubation 15 minutes. Temperature 33°C.

* Values given are for CO_2 evolved between 5 and 10 minutes after addition of substrate.

Acetate activating and condensing enzymes. Both the acetate activating and condensing enzymes have been shown to occur in crude extracts of *E. coli* by Novelli and Lipmann ('50). They concentrated these enzymes by fractionation with ammonium sulfate between 35 and 70% saturation. Essentially similar results were obtained in the present work. Crude extracts from *E. coli* were fractionated in small increments and each one tested for both enzyme systems. As was shown in table 1, the activities of both of these enzymes parallel each other and their specific activities increased by at least a factor of 2 after ammonium sulfate treatment. It is interesting to note that each ammonium sulfate fraction contained all of the necessary components for citrate synthesis.

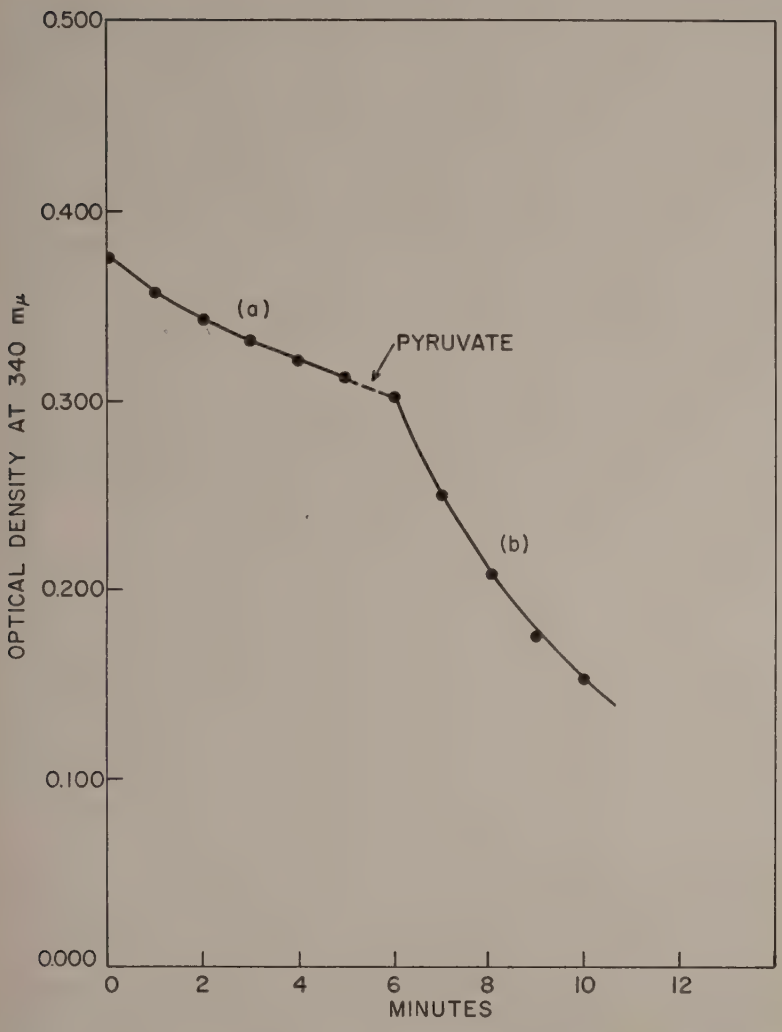

Fig. 7 Lactic dehydrogenase from *Escherichia coli*. Each cuvette initially contained 100 μM of phosphate buffer, pH 7.4 and 375 μg of protein obtained between 0.3 and 0.4 ammonium sulfate saturation. To the experimental cuvette was added enough reduced DPN to give a reading in optical density between 0.300 to 0.400. The rate of reduced DPN oxidation was determined (curve a). 5 μM of pyruvate were then added to each cuvette and reduced DPN oxidation again followed (curve b).

Lactic dehydrogenase. A reaction by which pyruvate can be shunted into the tricarboxylic acid cycle involves the conversion of lactate to pyruvate by the enzyme known as lactic dehydrogenase. Although this enzyme has been known to occur in *E. coli* since 1925 (Quastel et al., '25) and demonstrated in cell-free extracts by Stephenson in 1928 (Stephenson, '28) its cofactor requirements have not been established. That lactic dehydrogenase activity can be associated with DPN could be inferred from the data presented in figure 7 where the rate of oxidation of reduced DPN is greater in the presence of pyruvate than with reduced DPN and enzyme alone. This experiment was performed with 375 μg of protein obtained between 0.3 and 0.4 ammonium sulfate saturation where the bulk of lactic dehydrogenase was found. The slow oxidation of reduced DPN in the absence of pyruvate may be due to either a slow breakdown of the reduced DPN or the presence of minute amounts of substrate.

DISCUSSION

The data described in this report represent an attempt to correlate the occurrence as well as the relative concentration of the enzymes of the tricarboxylic acid cycle in any one bacterium. In addition, the association of the bulk of activity of the various enzymes with particular ammonium sulfate fractions may provide a convenient starting point for further purification and characterization of these enzymes. It is to be noted, however, that the association of a given enzyme with an ammonium sulfate fraction may vary slightly from one experiment to another and under different experimental conditions. In order to obtain reproducible results, it is imperative that such factors as pH and protein concentration of initial crude extracts be adequately controlled. In addition, working with bacterial extracts, the problem of viscosity becomes particularly cumbersome. This difficulty has been obviated by the treatment of such extracts with DNA'se which results in a reduction of viscosity by depolymerizing the DNA as well as in "clean" ammonium sulfate separations.

The concentration of the tricarboxylic acid cycle enzymes in crude cell-free extracts as well as in individual ammonium sulfate fractions has been determined. Aconitase appears to be present in least amounts whereas the most abundant enzyme was found to be malic dehydrogenase. On this basis, aconitase may be, in fact, one of the limiting enzymes of the cycle in *E. coli*. This supposition is based upon the assumptions that the optimum methodology was employed and that the procedure by which the cells were broken up did not result in significant destruction or inactivation of the individual enzymes. Furthermore, in some instances, as in the case of malic dehydrogenase and fumarase, the enzymatic activity was measured by the reverse as it would normally occur in the cycle. Consequently, the relative concentrations of these two enzymes may be somewhat different than those given in table 1.

SUMMARY

The distribution of the tricarboxylic acid cycle enzymes in *Escherichia coli* has been studied. By measuring the specific activity of these enzymes it has been possible to calculate their relative abundance in cell-free extracts as well as in individual ammonium sulfate fractions. Several of these enzymes have been partially purified and their cofactor requirements ascertained.

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SIMULTANEOUS EFFECTS OF METABOLIC INHIBITORS ON THE VISCOSITY, SURFACE RIGIDITY AND CLEAVAGE IN ILYANASSA EGGS

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FIFTEEN FIGURES

INTRODUCTION

With the centrifuge method Heilbrunn ('21) showed that viscosity fluctuations in the cytoplasm accompanied the cleavage of *Arbacia, Cumingia* and *Nereis* eggs, and were a primary factor in spindle formation. Heilbrunn and Wilson ('48) demonstrated a similar cycle in the cytoplasmic viscosity of Chaetopterus. The present interpretation by Heilbrunn ('52) of viscosity rise and cleavage is based on a protoplasmic clotting reaction enhanced by calcium. Carlson ('46) described a viscosity cycle somewhat resembling the above, in the neuroblasts of the grass-hopper. Other papers are pointed out by Heilbrunn and Carlson.

The viscosity or rigidity of the cortex was studied in *Arbacia* by Brown ('34) and in *Chaetopterus* by Wilson ('51). Marsland ('38, '39) using high hydrostatic pressure reversibly inhibited cleavage in *Arbacia.* He considered the cortex to become more rigid prior to cleavage while Wilson found the opposite to occur in *Chaetopterus.* It would be enlightening to study the effect of metabolic inhibitors (like azide, cyanide etc.) simultaneously on cleavage and on the colloidal state of cytoplasm and cortex. For one thing such study will in-

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dicate how significant viscosity is in cleavage. For another thing it may reveal unsuspected colloidal changes caused by these metabolic inhibitors, especially as the latter have become popular in studies connecting oxidative metabolism with cleavage. Partial inhibition of oxidation by such agents has been considered to block or delay cleavage (Spiegleman and Moog, '45; Runnström, '35; Clowes, '51; Fisher, '44). Might it not be that these agents interfered with the important sol-gel transformations during the mitotic cycle, and thereby blocked division?

A few papers related to this investigation are found in the literature. Beams and Evans ('40) found that a concentration of colchicine sufficient to arrest cleavage in *Arbacia* also decreased viscosity of the cytoplasm as shown by easier stratification upon centrifugation. The cortex, however, showed higher viscosity than the controls. Cheney ('49) using caffeine in concentrations that retarded *Arbacia* cleavage, did not find any evidence that the agent changed the viscosity; however, the surface became more resistant to deformation. Hultin ('50) found that iodoacetate caused a slight increase in the viscosity of egg homogenates. Runnström and Kriszat ('50) found ATP to increase the viscosity of the egg of *Psammechinus*.

MATERIALS AND METHODS

Eggs of the marine snail *Ilyanassa obsoleta* Stimpson (*Nassa obsoleta* say) were used. One capsule containing 60–80 fertilized eggs was employed for each experiment and not less than 20 experiments (20–70) were performed with each inhibitor. Periodically a polar lobe appears in the egg but the third round stage (lobe withdrawn) lasts long enough to make several centrifugations and take the necessary measurements on viscosity and surface rigidity. As only relative viscosity was measured no attempt was made to have a strictly constant temperature.

The centrifugal force used in most of the experiments was 1880 g, but occasionaly 5000 g was employed with Emerson

hand and electric centrifuges. The centrifugation duration was 5 minutes on the average but varied between 4–7. The medium of centrifugation was neutralized gum arabic made in 1:1 ratio in sea water.

Solutions of the inhibitors were made in sea water and the concentrations used were just sufficient to arrest cleavage reversibly, except for the cyanide, in which the cleavage was delayed, but not blocked. In some cases the pH was unaltered (pH of sea water, 8.1) but in cases where it was lower, a phosphate buffer was added and the sea water in the control group was treated similarly.

Procedure. The fertilized but uncleaved eggs were divided into two groups after removal from their capsule. One group was placed in the inhibitor for a certain period at the end of which (eggs still uncleaved) both this group and the control were centrifuged and their stratification zone measured with an ocular micrometer. The average width of the stratified zone in each group was taken as a criterion of relative cytoplasmic viscosity. Two other indices, which also vary directly with viscosity, were used: the time taken by each of the two groups to reach equal statification with the same force, and the centrifugal force needed to bring each group to the same stratification at the same time. A few eggs were left uncentrifuged to observe the effect of the inhibitor on cleavage; and occasionally fixed and stained preparations were made to compare the nuclear conditions. Photomicrographs were made with a Leica camera.

Preliminary experiments with one of the inhibitors (azide) indicated that the surface rigidity of the eggs was altered, for the eggs lost their spherical shape and upon centrifugation the treated eggs became easily deformed or elongated (see Harvey, '37). Hence it was necessary that all studies should include simultaneously measurements of cytoplasmic viscosity (as revealed by stratification) and of surface rigidity (as revealed by elongation). It was found that most of the agents increased the cytoplasmic viscosity. This would not tend to elongate the eggs, rather eggs that stratify sooner

will show elongation faster due to the pulling forces at the two poles. Hence in those agents that increased cytoplasmic viscosity, an elongation of the egg is not a secondary effect. of the pull at the poles.

RESULTS

Series A. Early immersion of eggs in the inhibitors

In this series the eggs were placed in the inhibitors as soon as they had completed the maturation divisions, and hence could remain in the agent for 50–60 minutes at the end of which they would begin to cleave.

1. *Effects of M/200 sodium azide at pH 6.6* (figures 1–4). By centrifuging at 10 minute intervals, a viscosity increase was detected within 10–15 minutes of treatment. After 45 minutes treatment the increase was estimated by the various criteria described under Methods and found to be at least 4 times that of the untreated eggs, placed in sea water buffered to pH 6.6. The latter were found not to differ from eggs in normal sea water. At the pH of sea water (8.1) azide also produced an increase in the viscosity, though to a lesser extent.

The surface effect was very striking. Eggs in the azide lost their spherical shape and flattened at one of the poles. Upon centrifugation these eggs became more elongated than the controls. The long diameters were in the ratio of 213 μ (treated) to 180 μ (controls). It should be emphasized that because the viscosity was lower in the controls, one would expect the control eggs to elongate much more due to the pulling action of the opposite poles. This makes the elongation of the treated and more viscous eggs an independent and genuine effect of the agent on the surface rigidity. Moreover the flattened appearance of treated eggs before centrifugation is an indication of a weakening of the surface forces.

The cleavage of the eggs in azide was reversibly blocked, for on return to sea water after one hour of treatment the eggs cleaved and showed normal gastrulation. The viscosity and surface rigidity also returned to normal.

2. *Effects of 0.003% 2,4,5-trichlorophenol at the pH of sea water* (figs. 11, 12, 13). Here again cytoplasmic viscosity increased. After 45 minutes of treatment it was estimated to be twice the normal.

The surface effect was like that of azide, for the treated eggs were flattened and on centrifugation elongated to 250 μ as compared to 200 μ of the controls.

The concentration used arrested cleavage reversibly even if treatment lasted several hours. The colloidal effects were also reversible.

3. *Effects of 0.1% monoiodoacetic acid at pH 6.4* (figs. 7 and 8). The cytoplasmic viscosity increased to twice that of the controls.

Effect on the surface was remarkable. Eggs lost their smooth round surface and became pointed and wrinkled. On centrifugation treated eggs became longer than the controls in the ratio of 205 to 180 μ.

The reversibility of the arrest of cleavage was less complete than in the previous agents, the treated eggs requiring 12 hours to recover and reach the 2-cell stage.

4. *Effects of potassium cyanide at pH of sea water.* There was no effect on cytoplasmic viscosity. The egg surface, however, was slightly more resistant to elongation: 176 μ (treated) to 193 (controls). Eggs cleaved in concentrations ranging from M/10,000–M/1,000, though with 20 minutes delay in the latter.

Clement ('40) had shown that *Ilyanassa* eggs cleave in cyanide. This places cyanide in a different category with respect to the other agents.

5. *Effects of 0.12% chloral hydrate at pH of sea water.* The treated eggs became less viscous in this agent and the magnitude was estimated as ½ the viscosity of the controls. The effect on the surface rigidity was not clear, probably indicating a lowering.

Cleavage was inhibited reversibly.

6. *Cytological findings.* The fixed and stained eggs showed that in all of the inhibitors except cyanide the nuclei remained

in the initial condition at which they were placed in the agents, i.e. the pronuclei stage, although these eggs remained for about an hour in the inhibitor. In control eggs and in cyanide the nuclei showed the usual mitotic phases.

TABLE 1

Summary of the action of the inhibitors on cytoplasmic viscosity and surface rigidity. Eggs centrifuged after long treatment (Series A).

	CYTOPLASMIC VISCOSITY (STRATIFICATION UNITS)		RECIPROCAL OF SURFACE RIGIDITY, I.E. ELONGATION. LONG DIAMETERS IN MICRA	
	Treated	Controls	Treated	Controls
Azide	4	1	213	180
Trichlorophenol	2	1	250	200
Iodoacetic acid	2	1	205	180
Potassium cyanide	No change		176	193
Chloral hydrate	½	1	Not clear	

Series B. Late, and short treatment

The question naturally arises, what would happen if the eggs were placed in the inhibitors for only a short duration selected close to the expected cleavage time? The experiments to be reported below show that such eggs succeeded in cleaving in spite of the viscosity rise or fall as the case may be. The effect on the surface rigidity was not very distinct.

1. *Effects of M/200 azide at pH 6.6* (figs. 5 and 6). Eggs were placed in this agent after they had given the third polar lobe for periods varying between 15–20 minutes. They were able to divide on time with the controls. Centrifugation prior to and in the two-cell stage with the controls showed that treated eggs had the higher viscosity expected of azide treatment. Treated eggs also manifested a tendency to elongate more readily than their controls indicating a weakening of surface rigidity.

2. *Effect of 2,4,5-trichlorophenol* (figs. 9 and 10). Eggs placed 15–20 minutes before the expected cleavage time were able to divide with the controls. On frequent centrifugation prior to and in the two-cell stage they showed the expected increase in cytoplasmic viscosity. The surface elongation was also slightly higher than the controls, as in the first series.

3. *Effect of chloral hydrate* (figs. 14 and 15). Eggs placed in this agent 15–20 minutes before expected cleavage, reached the two-cell stage with the controls. All such divided eggs showed the expected lowered viscosity when centrifuged for 4 minutes with their controls. Thus cleavage took place in spite of the lowered viscosity. Centrifugation of treated eggs at 5 minute intervals during the treatment period did not show any change in the lowered viscosity. The latter seemed to remain low during treatment and division time.

DISCUSSION

Table 1 summarizes the action of the 5 agents on cytoplasmic viscosity and surface rigidity. Cyanide which does not block cleavage in *Ilyanassa* (in agreement with the frog's eggs and in contrast with the sea urchin's) did not alter the viscosity, although a slight surface change was indicated. The other agents which affected cleavage changed the viscosity and surface rigidity. Three of them increased viscosity and one decreased the surface rigidity; the last decreased viscosity and did not have a clear action on the surface.

This work indicates an action of 5 known metabolic inhibitors on the colloidal properties of the cytoplasm and cell surface. Hence interpretations by investigators based wholly on the respiratory inhibition of the agents should be made cautiously (see Introduction). Possibly some kind of colloidal change either in the surface or deeper cytoplasm, prolonged to about an hour after maturation divisions (the usual interval in the experiments employing respiratory techniques) may be involved in the cleavage inhibition.

Returning to the specific action of the agents, azide, trichlorophenol and iodoacetate increased cytoplasmic viscosity but lowered the surface rigidity. Heilbrunn ('52) refers to many instances where a decreased viscosity of the cortex was associated with an increased cytoplasmic viscosity but no clear explanation of the mechanism of this paradoxical state is given. The present author visualizes an interpretation based on denaturation of the egg proteins. It is known that the viscosity of a protein solution is related to the size and shape of its molecules and that coiling and uncoiling of such molecules will alter the viscosity (West and Todd, '51). Denaturation of the proteins in the egg cytoplasm by the inhibitors could have produced the viscosity increase. The protein chains of the surface membrane would also be denatured, but the effect of the denaturation would be different because the arrangement of the protein in the membrane is peculiar. Here the partly coiled fibers that hold the membrane would be uncoiled. The straightened chains would be arranged meridionally and hence their strength in holding the surface will be much reduced resulting in easier elongation upon centrifugation. Thus the seemingly paradoxical situation can be explained on the basis of one effect, i.e. denaturation, if one takes into consideration the architecture of the protein chains in the membrane as contrasted with those in the cytoplasm. That the proteins in the membrane are "fibrous" or in chains is often acknowledged, and a recent support is given by Mitchison ('52): "if the bonds holding the protein chains in the looped configuration are broken, the protein chains will come to lie more in the plane of the surface through random agitation. The thickness of the membrane will diminish, but there will be a corresponding increase in the surface area."

The finding that eggs divided in spite of the viscosity rise or fall upon short treatment before the expected cleavage time is significant in view of the role usually attributed to viscosity in bringing about cleavage. Of course viscosity rise in very early stages may be related to spindle formation,

and its suppression may arrest mitosis. But we are now concerned with the viscosity change just prior to the cleavage furrow. In *Ilyanassa* the viscosity fluctuates from high during the round stages to low during the polar lobe stages. It also has a very slight rise of short duration prior to cleavage. The short treatment showed that whether the viscosity rose (azide, etc.) or fell (chloral hydrate) it did not hinder the cleavage furrow from going to completion. It is important to determine the course of events under such treatment in the sea urchin where the rise in viscosity is sharp and continues during cleavage. If the sea urchin eggs divide in spite of their lowered viscosity in chloral hydrate, then cytoplasmic viscosity rise can not be instrumental in bringing cleavage. Probably this will favour the cortex, see again Mitchison ('52). The author is now investigating the problem in the sea urchin.

The cytological studies showed that mitotic phases were blocked during long treatment in these agents. Whether it was a denaturation effect or a sequel to energy suppression is not known although attempts to restore mitosis by adding ATP failed. At any rate there was a correlation between nuclear block and cleavage block such that if the nucleus did not enter its mitotic phases the cleavage process did not get started. This is in line with Cornman and Cornman ('51) and Swann ('52) who postulate a nuclear or chromosomal agent that initiates cleavage.

SUMMARY

A simultaneous study of the effect of metabolic inhibitors on cleavage, cytoplasmic viscosity and surface rigidity was attempted.

1. M/200 sodium azide (pH 6.6) inhibited mitosis and cleavage reversibly. The viscosity was increased but the surface rigidity was lowered. These effects were also reversible on return to sea water.

2. 0.003% trichlorophenol (pH of sea water) had similar effects to azide, though viscosity rise was smaller in magnitude.

3. 0.1% iodoacetic acid (pH 6.4) had the same general effects of azide and trichlorophenol.

4. Potassium cyanide (M/10,000–M/1,000) did not inhibit cleavage, did not change the viscosity, but caused a slight increase in surface rigidity.

5. 0.12% chloral hydrate (at pH of sea water) blocked cleavage, lowered the viscosity but did not have a clear effect on surface rigidity. All effects were reversible.

6. When eggs were placed in the various inhibitors for a short duration close to the expected cleavage time, they were able to divide although the viscosity change (increase or decrease) took place.

It was concluded that metabolic inhibitors alleged to block division through energy interference, also have colloidal effects that may be involved in the arrest of cleavage.

A hypothesis based on protein denaturation was given to explain the paradoxical rise in cytoplasmic viscosity with the fall in surface rigidity. The finding that eggs divided in spite of viscosity change was taken to disfavour current opinion that cleavage is instrumented through a rise of cytoplasmic viscosity just prior to the appearance of the cleavage furrow.

ACKNOWLEDGMENTS

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PLATE 1

EXPLANATION OF FIGURES

1 Eggs centrifuged at 1180 g for 7 min. after 45 min. treatment in M/200 azide, showing rise in viscosity and fall in surface rigidity.

2 Controls of figure 1.

3 Same treatment as in figure 1 from another experiment.

4 Controls of figure 3.

5 Cleaved eggs after azide treatment showing high viscosity.

6 Controls of figure 5.

7 Centrifuged eggs after treatment in iodoacetate showing increased cytoplasmic viscosity and decreased rigidity of surface.

8 Controls of figure 7.


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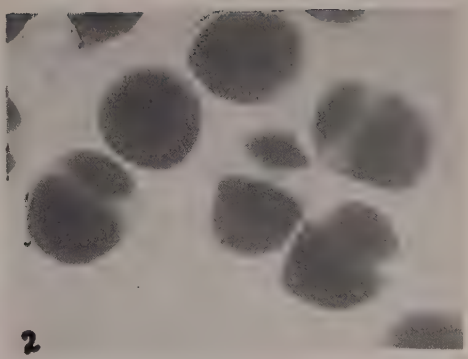
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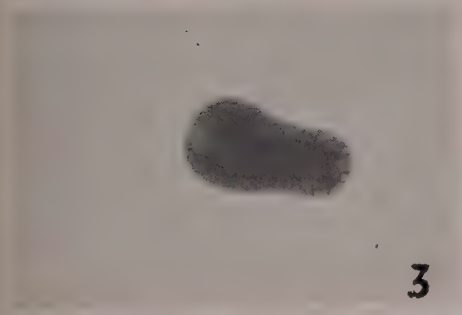
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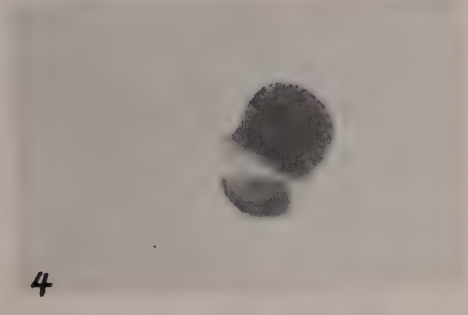
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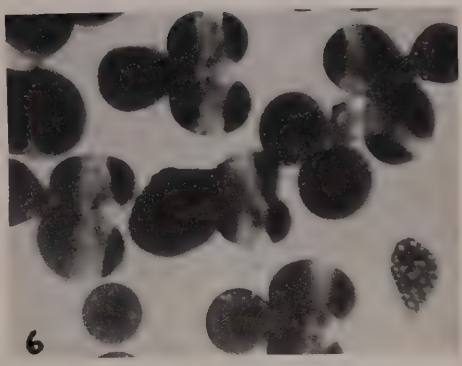
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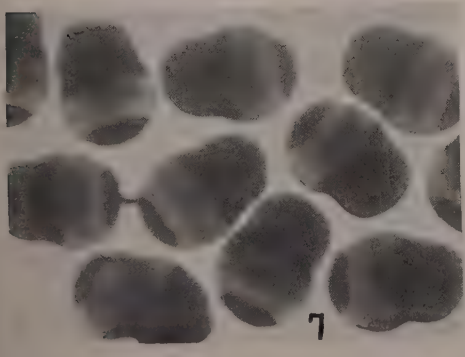
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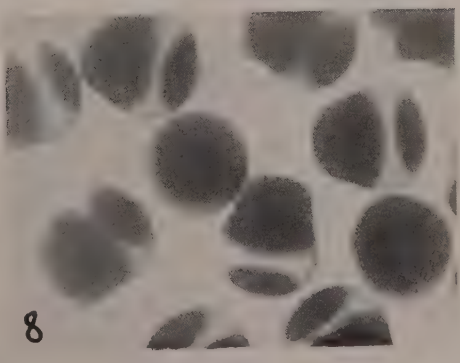
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PLATE 2

EXPLANATION OF FIGURES

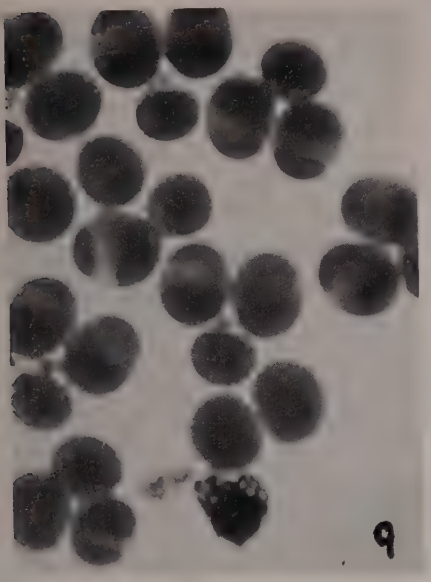
9 Cleaved eggs after trichlorophenol treatment showing high viscosity.

10 Controls of figure 9.

11 Flattened (uncentrifuged) eggs due to treatment in trichlorophenol.

12 Centrifuged eggs after treatment in trichlorophenol showing increased viscosity and decreased surface rigidity.

13 Controls of figure 12.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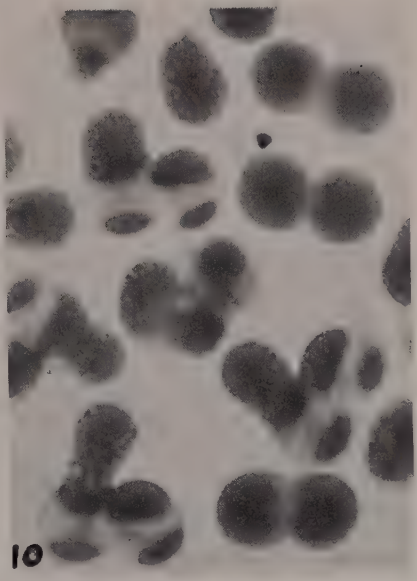
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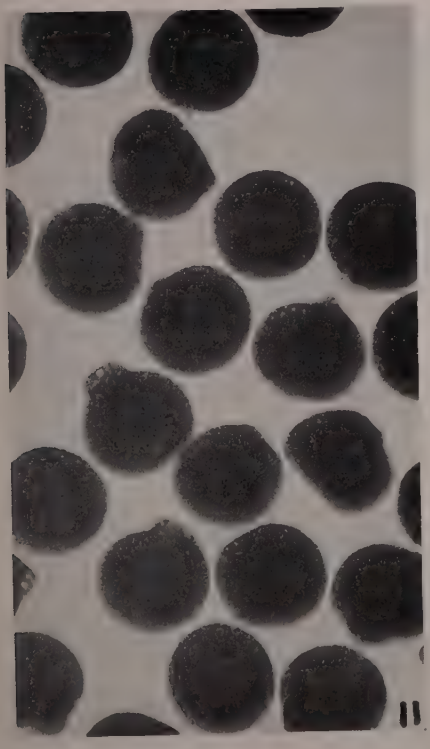
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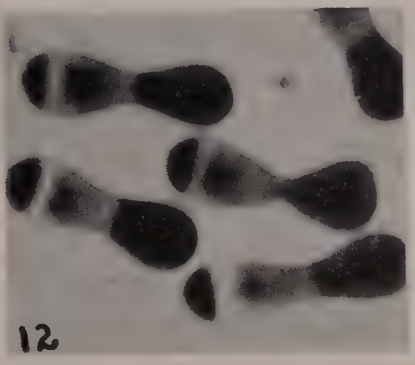
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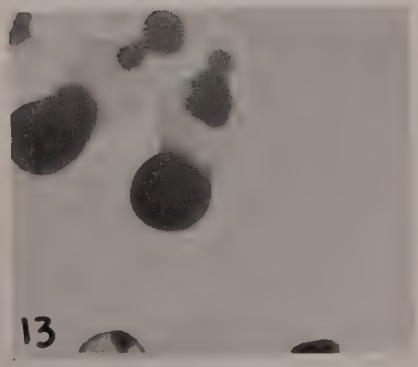
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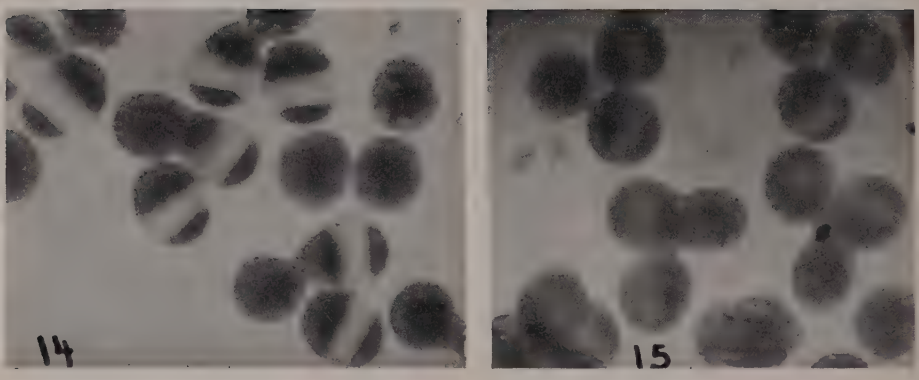

14 Cleaved eggs after chloral hydrate treatment centrifuged for 4 minutes at 1880 g showing lowered viscosity.

15 Controls of figure 14.

TEMPORAL STUDIES OF CELL DIVISION

I. THE INFLUENCE OF PLOIDY AND TEMPERATURE ON CELL DIVISION IN S. CEREVISIAE [1]

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SEVEN FIGURES

INTRODUCTION

The quantitative growth and division characteristics of living cells have generally been determined in the past from a consideration of the average activities of a large population of cells. Microorganisms are easily grown en masse in liquid media and the increases in number of cells, dry weight, nitrogen content, etc., can be followed; from these data the average rate of cell division may be determined. The observation of average population behavior is of course very fruitful, but frequently misses important information. To the author's knowledge very few investigations of the distribution of division times of individual cells and of the relation of the division time of a mother cell to that of its progeny have been undertaken. ("Division time" is used here to mean intercleavage time as contrasted to the time spent in actual mitosis.) Blum ('50) studied the time relationships in the cleavage of the normal fertilized egg of *Arbacia punctulata;* the distribution of division times was found to approach normal (Gaussian) and a deviation from the mean division time in one generation was not passed on to the descendants of the deviating cell. Kelly and Rahn ('32) studied the growth rate of individual bacterial cells through four generations. They

[1] This work was partially supported by a contract between the University of California and the Atomic Energy Commission.

found that growth-rate variation was not heritable and that the division-time distributions were slightly skewed; a mathematical theory of cell division was developed on the basis of the distributions.

The investigation reported herein was undertaken in an effort to learn something of the mechanisms underlying cell division in yeast and to provide a basis for the study of radiation-induced division delay. The question whether the total amount of genetic material that must be synthesized for each division of cells of a given species is the paramount rate-limiting factor for cell division under optimal growth conditions can be investigated in yeast, since related haploid, diploid, triploid and tetraploid strains are available. Following individual cells and their progeny through many generations makes it possible to determine the heritability of division-time deviations, to determine if the degree of correlation between the division times of the two cells resulting from a division is higher than that among the population as a whole, and to determine the effect of temperature on synchronously dividing cells. These results are used to consider the relative roles of genetic material and other protoplasmic entities as pacemakers for cell division. This last problem was attacked by Moore ('33), who fertilized nucleate and enucleate eggs of *Dendraster* with either *Dendraster* sperm or *Strongylocentrotus* sperm; the fertilized eggs cleaved at a rate typical of *Dendraster* in every case, from which Moore concluded "The reactions of the cytoplasm alone determine the rate of cleavage, even when the fertilizing sperm is from an animal of different order." On the other hand, Blum ('51) found that division delay in the eggs of *Arbacia* caused by ultraviolet or X-radiation was due to nuclear damage. These two opinions are not necessarily contradictory, but there is clearly need for more information on the problem.

It should be pointed out that the process of cytokinesis in yeast is rather unusual. The cells divide by budding rather than by forming a furrow or cleavage plate. The bud appears first as a tiny knob on the cell wall and then gradually grows

into a full-sized cell. There is evidence (Spiegelman, '51; Ephrussi, '51) that the particulate contents of the mother are shared equally with the daughter, at least in diploid cells growing at the optimal temperature of 30°C. The relationship between cytokinesis and karyokinesis is not accurately known, partially because there is no universal agreement as to what structure in the cell is the nucleus and also because it is impossible to recognize stages in mitosis unequivocally. Delamater's ('50) pictures of stained material show buds that are half the size of the mother cell before nuclear material enters them. The latter result has been confirmed by Mortimer ('54) on the yeast strains used in this work.

METHODS AND MATERIALS

Five different strains of *Saccharomyces cerevisiae* were used: SC6, SC7, X30, X31, X33. SC6 and SC7 are diploid and haploid strains respectively; they have been described by Zirkle and Tobias ('53). X30, X31, and X33 constitute a genetically related polyploid series of *Saccharomyces*; they have been described by Mortimer ('53). X30 is diploid, X31 is triploid, and X33 is tetraploid. The diploid X30 was used in the majority of studies to be reported.

Stock cultures were carried on YED [2] slants in 5 cm^3 screw-cap vials at 2° to 4°C. Each month a new slant was inoculated by means of platinum-loop transfer from the previous slant, incubated at 30°C. for 48 hours, and then refrigerated. As needed, subcultures were prepared from the stock cultures and used experimentally within two days after the end of incubation.

In order that the division times of individual cells may be followed through many generations these conditions must be met:

1. The cells must be available for continuous observation.
2. The temperature of the cell's environment must be constant during the course of an experiment, so that temperature fluctuations will not cause fluctuations in generation time.

[2] YED is ½% yeast extract (Difco), 1% dextrose, 2% bacto-agar (Difco).

3. The cells must be in a constant nutrient environment.

4. The cells must be fixed in the field of observation to permit individual identification, yet they must be available for manipulation to allow the separation of successive generations. It is necessary to separate daughter cells from mother cells because the times, both at birth and at division of any given cell, must be known in order to determine its division time; hence, each cell must be uniquely identified. This is difficult in a group of more than 6 or 8 yeast cells. Furthermore, the cells in a large group do not have a uniform nutrient environment. Hence, a micromanipulator to permit the selection and separation of cells under observation is necessary.

The conditions above can be met by the use of a moist chamber in conjunction with an agar-coated cover slip. The nutrient medium used in all experiments is one that is optimum for yeast growth — $\frac{1}{2}$% yeast extract (Difco), 1% dextrose, 2% bacto-agar (Difco). This mixture was autoclaved and distributed among small screw-cap vials for storage. When needed, one of these vials was heated in boiling water and one drop of the medium was put on a cover slip (lucite cover slips were used). The drop was spread with a platinum loop to secure an even layer of agar about 0.5 mm thick.

Cells from a subculture 2 to 4 days old were suspended in M/20 KH_2PO_4 buffer at a concentration of about 5×10^6 cells/ml. With the aid of a small nichrome loop, a drop of this suspension was streaked on the surface of the cover-slip agar. (In some experiments two different strains were observed simultaneously for comparison purposes. With care, it was possible to place a second streak within 0.5 to 1 mm of the first.) The streak dried quickly, after which the cover slip was placed on the moist chamber, agar side down.

A microscope equipped with a special micrometer slide holder was employed. The slide holder, carrying the moist chamber, can be moved smoothly through distances as little as 1 or 2 μ. The micromanipulator is a simple but very efficient device which imparts motion in three directions to the yoke holding the microneedle by means of fine screws. The micro-

needle is a glass rod about 10 cm long. One end is drawn to a fine point, and the last centimeter of it is bent at an angle of about 60°. The point is sharpened by dipping it into concentrated hydrofluoric acid and withdrawing slowly (after a method of S. E. Reaume). In this manner a point of 2 to 5 μ in diameter can be made.

In order to provide constant temperature conditions the entire illuminator-microscope-micromanipulator assembly is enclosed in a temperature-regulated box with a transparent top. In operation, the temperature of the box does not fluctuate by more than $\pm$ 0.5°. Screened holes in the box permit micromanipulation to be carried on without disturbing the temperature.

Yeast cells prepared as described, when transferred to YED agar, have a latent period of several hours before they begin to divide, even at the optimum temperature of 30°. Hence, there was sufficient time after introduction of the moist chamber and agar-coated cover slip for these to reach the regulator-controlled temperature before the first cell divisions.

When about half the cells had divided, one budding cell was chosen and was moved onto a clear portion of the agar with the microneedle. Cells seemed to adhere readily to the microneedle and usually followed closely in the wake of the microneedle as it moved through the liquid surface film of the agar. On several occasions, deliberate attempts were made to injure a cell with the microneedle by poking and rubbing; in no case did this treatment cause morphological changes in the cell or delay of division.

In all strains of yeast studied, it was found that mother and daughter cells adhered tightly to each other and could not be pulled apart with the microneedle. As soon as either mother or daughter began division, however, they could be separated easily. Hence, the initial cell chosen for study was allowed to form a "double" (two attached mature cells), and when the second generation appeared the budding mother cell and the budding daughter cell were separated. Succeeding

generations were separated in the same manner; no two cells were ever closer to each other than 30 to 50 μ.

The problem of keeping track of the position and time at budding of each individual cell becomes serious in the fifth and sixth generations (in the sixth generation there are 32 cells with 32 buds), so a system of positioning and tabulation had to be evolved. The system of positioning is illustrated in figure 1: The initial cell is placed in position b; the first cell to produce a second-generation bud is moved to c. The

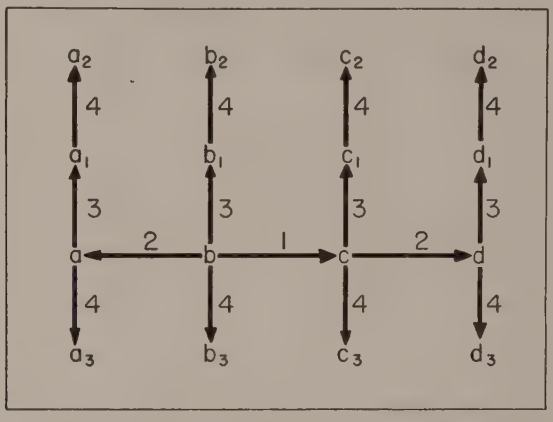

Fig. 1 Diagram of the method of positioning successive generations. Initially there is a double at position b; when the double buds one budding cell is left at b, and the other is moved to c. This constitutes separation 1, as indicated by the numeral 1 appearing above the appropriate arrow. Movement of successive generations to appropriate positions is accomplished in a similar manner.

third-generation budding cells occupy positions a, b, c, d, and so on as indicated by the arrows in figure 1 until all sixteen positions are occupied in preparation for the sixth generation. All observations were carried through the sixth generation. In preliminary tests it was shown that moving a cell did not predispose it to divide earlier or later than the stationary cell; hence the convention of moving the first cell to bud at a given position did not cause systematic error.

Under the experimental conditions outlined above, at 30°C., yeast cells divide once every 70 minutes or so; the width of

the distribution of division times is about 15 minutes. It is evident that observations must be made every few minutes to obtain an accurate distribution. This brings up the question of just how accurately the onset of budding can be determined. The strains of yeast employed were of the "nonsticky" variety, which seems to have something to do with the fact that budding generally occurred in a horizontal direction; i.e., in the plane of the agar. Buds are clearly delineated within 5 minutes after the onset of budding; in order to minimize the effect of operator decisions as to whether a bud was definitely present or not, observations were made at 5-minute intervals. A bud that was scored at the end of a given 5-minute interval was plotted for histogram representation as belonging within the interval preceding the scoring time.

Although photomicrographic equipment was available for recording the appearance of buds, it was found that unequivocal identification of a 5-minute bud sometimes required up-and-down focusing, so that direct observation and recording were more accurate. The time used at the end of each interval for observation and recording had to be short, however, in order to avoid continuous observation. To satisfy this requirement division times were recorded on a two-dimensional pattern corresponding geometrically to the pattern of cell positions. With this method of tabulation the time required for observation and recording was about a minute or less. All observations were made at 430 $\times$ total magnification.

RESULTS

Distribution of division times of diploid cells

The optimum growth temperature for many yeasts, including the strains used in this investigation, is 30°C. For this reason the characteristics of division at this temperature are of practical importance and were investigated most extensively. The strains SC6 and X30 were both employed; since X30 was the strain used in radiation division-delay work it was investigated in more detail than SC6. It is of interest to

note that the division time distribution of SC6 is nearly the same as that of X30.

The results contained in three division schedules were pooled to plot the series of histograms shown in figure 2. The number of divisions per 5-minute interval is plotted on the ordinate, and the abscissa gives the generation number, each generation being divided into an appropriate number of 5-minute intervals. It can be seen that the average division time does not vary with generation. It is important to establish this fact in order to be sure that there is no gradual

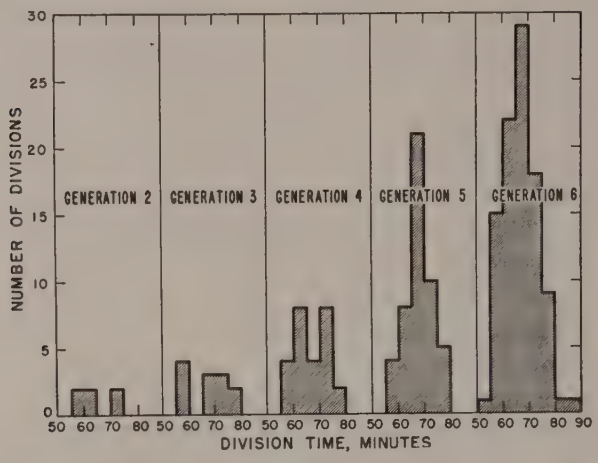

Fig. 2 Distributions of division times for various generations. Diploid yeast at 30°C.

adaptation of the cells to their environment over a period of several generations. Since the results from successive generations are equivalent, they may be pooled. This has been done to plot figure 3, which is also normalized. The data used to plot this figure comprise the division times of 186 cells. The mean division time is 67 minutes and the standard deviation is 6.4 minutes. (This is the standard deviation of the population, not the mean, so it is a measure of the width of the distribution.) It is notable that no dead or senescent cells appeared during six generations of growth. It was further ob-

served that the mother and daughter cells composing a "double" nearly always divide simultaneously at 30°C. in diploid strains (fig. 4).

At 20°C., X30 doubles divide synchronously just as at 30°C. The division times of 87 cells were observed. The percentage of divisions occurring in 5-minute intervals is plotted in figure 3. It can be seen that the distribution is much wider than the one at 30°C. The mean division time is 162 minutes, the median is 158 minutes, and the standard deviation is 15.4 minutes.

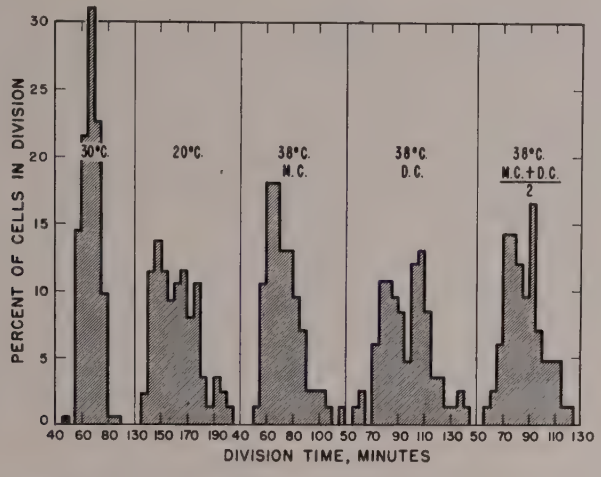

Fig. 3 Distributions of division times of diploid yeast at 20°, 30°, and 38°C. M.C. is mother cell, D.C. is daughter cell.

At 38°C. a new phenomenon appeared; the mother and daughter cells constituting a "double" did not divide synchronously. It was further found that the daughter was always the last to bud. The population of cells existing in any given generation was evidently not homogeneous, so the division times of mothers and daughters are plotted separately in figure 3. For the mother cells the mean division time is 73.5 minutes, the median is 69 minutes, and the standard deviation is 13 minutes. The striking fact about this distribution is that although the mean is only 6.5 minutes greater than the

30°C. mean, the distribution is about twice as wide. (The data presented are taken from 6 sixth-generation division schedules, each of which shows the same broad distribution as the pooled data. A total of 168 cells was observed.)

For the daughter cells the mean division time is 96.5 minutes, the median is 93 minutes, and the standard deviation is 18 minutes. The daughter division time is then 23 minutes longer on the average than the mother division time; the distributions of mother and daughter division times are of comparable width, however.

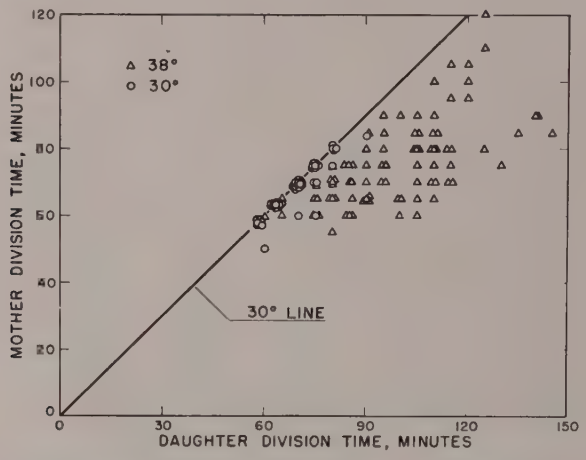

Fig. 4 The regression of mother division time on daughter division time for diploid cells at 30°C. and 38°C. The 30° data includes 41 points falling on a line of 45° slope, labeled the "30° line" in the figure. All points on this line represent synchronous division of mother and daughter.

To obtain an estimate of the effect of 38°C. on mother and daughter cell considered as a unit (i.e., to average the asymmetric effect of 38°C. on mother and daughter), the division times of each pair were added, divided by 2, and grouped into 5-minute intervals. The resulting histogram is plotted in figure 3. The mean is 84 minutes, the median 82.5 minutes, and standard deviation 14 minutes.

Figure 4 shows daughter division time plotted versus mother division time for each pair. Whereas the 20° and 30°

data fall on a straight line of 45° slope (only the 30° data are plotted in fig. 4) the 38° data are widely scattered, illustrating the lack of synchrony at this temperature.

Distribution of division times of haploid, triploid, and tetraploid strains at 30°

One complete division schedule plus a sixth-generation replication for each ploidy was secured at the one temperature, 30°C. These experiments were sufficient to show that any

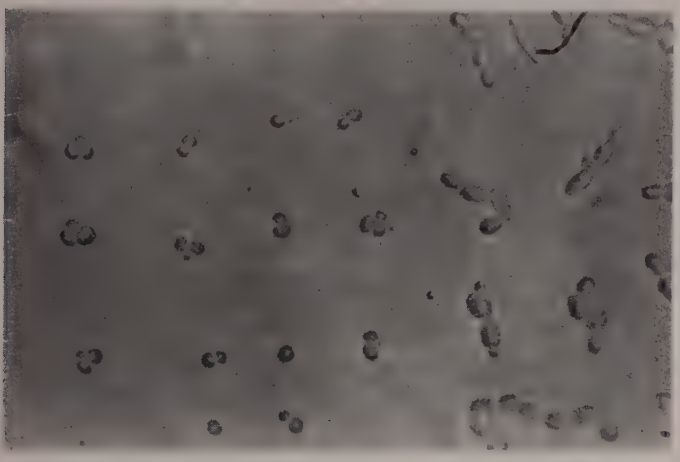

Fig. 5 Haploid and tetraploid cells in the sixth generation. The small cells on the left are haploid, the large cells on the right are tetraploid. It can be seen that the haploid doubles are budding asynchronously, whereas the tetraploid doubles have budded synchronously.

variation of division time with ploidy could only be very small, with the exception of the haploid. The triploid and tetraploid distributions were similar in shape and width to the X30 distribution.

Mother and daughter cells of the haploid SC7 did not divide synchronously; this is in contrast to the behavior of the two diploids, the triploid, and the tetraploid (fig. 5). The mean division time of the mother cells was 69 minutes and of the daughter cells 97 minutes. A correlation plot is presented in figure 6. It is seen that some SC7 doubles budded out of

synchrony by as much as 65 minutes. This behavior is qualitatively very similar to that of the diploid at 38° (see table 1, summary of results). The mother-cell division time is similar to that of the other ploidies at 30°C., but the daughter division time is much longer. The mean time of mothers and daughters is 83 minutes, compared to 84 minutes for the same quantity calculated for diploids at 38°C. Kilkenny ('52), using light-absorption methods to measure the growth of yeast in liquid media, found that his haploid strains had a generation time of about 160 minutes in "full rich medium," whereas the

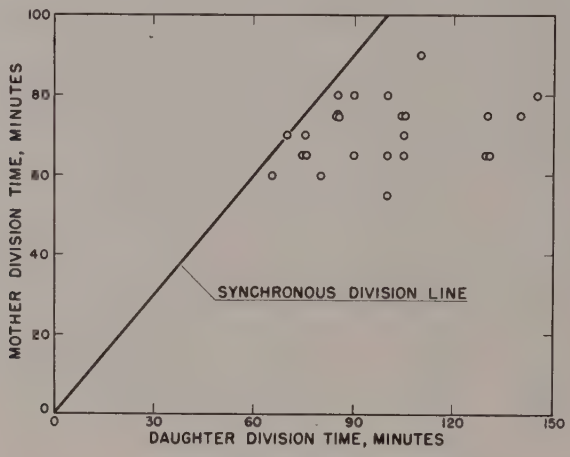

Fig. 6 The regression of mother division time on daughter division time for haploid cells at 30°C.

diploids took about 130 minutes. The ratio of these times is 160/130 or 1.23, which agrees with the ratio found here, 83/67 or 1.24.

The triploid, X31, had a mean division time of 68 minutes and it budded essentially synchronously. No division peculiarities were observed, except that one double out of the 31 observed took 190 minutes to divide.

The tetraploid X33 had a mean division time of 62 minutes and it budded synchronously.

A summary of the data for all ploidies is presented in table 1.

TABLE 1

Summary of results

PLOIDY	TEMPERATURE	MEAN DIVISION TIME	HALF-WIDTH OF DIVISION TIME DISTRIBUTION [1]	SYNCHRONOUS DIVISION OF MOTHER AND DAUGHTER
		mins.	*mins.*	
Diploid	20°C.	162	10.0	yes
	30°C.	67	4.3	yes
	38°C. Ave.[2]	84	9.4	no
	M. C.	73	8.7	
	D. C.	96	12.0	
Haploid	30°C. Ave.[2]	83	..	no
	M. C.	69	..	
	D. C.	97	..	
Triploid	30°C.	68	..	yes
Tetraploid	30°C.	62	..	yes

[1] Half-width is 0.67 times the standard deviation.

[2] Calculated by averaging mother cell (M.C.) and daughter cell (D.C.) times, as explained in the text.

Inheritance of division time

The data show no significant inheritance of division time in any generation. This is in agreement with the results of Blum for sea urchin eggs ('50). A correlation plot of fifth-generation division time versus sixth-generation division time is presented in figure 7. The ordinate value of each point represents the fifth-generation division time of a single cell, and the abscissa value represents the sixth-generation division times of the two cells formed in the fifth generation. (The division times of two cells can be plotted at one abscissa value because diploid doubles at 30°C. divide synchronously.) It can be seen that the only evidence of correlation occurs near the mean division time, where an apparent correlation would

be excepted to occur by chance alone. The wide scattering of points both horizontally and vertically is evidence for the hypothesis that deviation from mean division time is not heritable, even through a single generation.

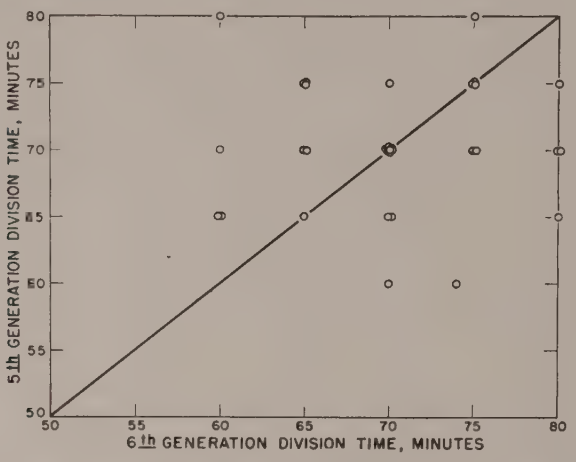

Fig. 7 The regression of 5th generation division time on 6th generation division time, for diploid cells at 30°C.

DISCUSSION

The 4 ploidies investigated — haploid, diploid, triploid, tetraploid — contain genetic masses in the proportions 1:2:3:4, respectively. It has also been shown that the cellular volumes (Mortimer, '53), the dry weight per cell, RNA per cell, and DNA per cell (Ogur, '52, using another polyploid series) of these ploidies stand in approximately the same relation to one another, 1:2:3:4, respectively. If division time were strongly dependent on the average *amounts* of protein, RNA, DNA, or chromosomal material per cell, we would expect to find large division time differences among different ploidies. Since such differences do not appear, it may be that the proportions rather than the amounts are important in determining division time. The fact that protein and RNA increase in proportion to genetic mass while the division time,

at least in the case of mother cells,[3] remains essentially constant suggests that increased RNA or protein compensates for increased genetic material. Such a compensation might come about, for example, if a fixed amount of RNA, or, perhaps, of an energy-rich compound, were required for the reproduction of each unit of genetic material. James ('53) has shown that RNA is rapidly synthesized in amebae just before division, which suggests that it triggers cell division.

Further information on the process of cell division in yeast is provided by a consideration of the data on synchrony. Although division time variations are not passed from one generation to the next, there is one case where the time variations of two members of the population are the same: In the diploid at 30° the mother and daughter cells of a double divide synchronously. On the other hand, in the diploid at 38°C. and in the haploid the mother and daughter cells of a double divide asynchronously. It is apparent that the factor determining division time is distributed equally to mother and daughter in the diploid at 30°C., but that it is distributed unequally in the haploid and in the diploid at 38°C. Further, in the latter case the mother cell always has a shorter division time than the daughter cell, so the mother apparently keeps a lion's share of the factor determining division time. Since the genetic material must be equally distributed, we conclude that the factor that controls the rate of normal division is extrachromosomal. What is the nature of this factor? It is observed that the daughter cell of a diploid double at 38°C. is usually smaller than the mother cell at the time the latter divides; we might, therefore, speculate that cell division takes place when a certain critical *total* volume of cytoplasm is exceeded. This idea fits the case for amebae, where division may be delayed indefinitely if the ameba is prevented from growing in volume by periodic excisions of the cytoplasm. On the other hand, yeast cells of a rather large range of diameters

[3] Recent work by Dr. Mortimer has shown that all ploidies are cytochrome positive, with the possible exception of the *haploid*. This may be the reason for the relatively slow division of the haploid daughters.

are observed to bud (observations of the author; also Lindegren and Haddad, '54), so it is logical to suggest that division occurs when some particular component of the cytoplasm, which is not in constant proportion to the total amount of cytoplasm, exceeds a critical value. Spiegelmann et al. ('51), working with yeast at 30°C., have demonstrated the equipartition of certain cytoplasmic particles during division. They showed that the presence of a galactose-positive phenotype required the cell to have at least one galactose-positive particle; by considering the manner in which galactose-positive phenotypes disappeared during cell division they were then able to show that the probability for any given galactose-positive particle to pass into the daughter cell during division was just one-half. Unfortunately, the distribution of particles at 38°C. was not investigated. Nickerson ('54) has found that yeast cells growing in the absence of cell division (filamentous growth), due either to mutation or starvation, exhibit tetrazolium reduction but are otherwise normal with respect to metabolic activities. These and other data are interpreted to mean that a reaction essential for cell division is coupled via an oxidation-reduction at a flavoprotein locus to cellular metabolism. Microscopic examination revealed that the site of tetrazolium reduction (and presumably of the flavoprotein locus) is on certain large mitochondrial granules of the yeast cell. On the basis of the present work, it is logical to suggest that the division time of a given cell is inversely related to the initial concentration of division-specific loci in that cell. (The use of "concentration" instead of "amount per cell" allows the above formulation to apply equally well to the cells of different ploidy.)

It is of interest to note that the synchronous division of mother and daughter diploid cells at 30°C. implies that they are functionally connected until preparation for the succeeding division is complete. This conclusion is supported by the observation that mother and daughter cannot be pulled apart ordinarily until they bud. Mundkur's ('54) pictures of sec-

tioned budding cells show that the cytoplasms of mother and bud are usually interconnected until the bud is quite mature.

The bearing of this work on certain mathematical theories of cell division is worthy of mention. Rahn ('32) assumed that the rate of gene reproduction in a population is proportional to the number of unreplicated genes, and that any given cell divides when all its genes have reproduced. Rahn derived an expression relating the distribution of division times to the probability of reproduction per gene per unit time and to the number of genes. It can be shown mathematically that mean division time depends strongly on probability of reproduction as defined by Rahn, but that the width of the division-time distribution changes only infinitesimally with large changes in mean division time. Hence this theory is not in agreement with the data reported herein, even if the reproducing units are considered to be cytoplasmic particles instead of genes. Kendall ('48) considered a multiple-phase birth process: When a new individual is born it passes through a series of phases, k in number, and only after it has attained the kth phase can it undergo division. The lifetime in each phase is assumed to be distributed exponentially and to be independent of the lifetimes in other phases. Kendall has analyzed the data of Kelly and Rahn on *Bacterium aerogenes* and has obtained values of k ranging from 12 to 47. The generation time has the distribution of $\frac{1}{2k\lambda} X^2_{2k}$, where $\frac{1}{\lambda}$ is the mean generation time, and X^2_{2k} is a chi-square variate of 2k degrees of freedom. The standard deviation of this distribution is $\frac{1}{2k\lambda}$ S.D. $(X^2_{2k}) = \frac{(4k)^{\frac{1}{2}}}{2k\lambda} = \frac{1}{\lambda k^{\frac{1}{2}}}$. The values of k characteristic of division of yeast at various temperatures can be determined from this formula if the experimental sample standard deviation is equated to $\frac{1}{\lambda k^{\frac{1}{2}}}$ and $\frac{1}{\lambda}$ is equated to the experimental mean generation time.

Temperature	20°	30°	38° (M.C.)	38° (D.C.)
Experimental mean generation time	162	67	73	96
Sample standard deviation	15.4	6.4	13	18
k	111	110	31	29

The table shows that the number of phases does not change with temperature between 20° and 30°, but the length of time spent in each phase is greater at 20° than at 30°. At 38° the number of phases is greatly reduced; mother cells and daughter cells have the same number of phases even though their means are different. Since the number of phases varies with temperature, the phases cannot correspond to elements of chromosome structure, unless the radical assumption is made that the number of distinct elements can vary. No matter what the phases might correspond to, it is hard to see how mother cells and daughter cells at the same temperature of 38° could have the same number of phases and yet different mean generation times. The multiple-phase theory apparently does not afford a simple, convincing explanation of the present data.

ACKNOWLEDGMENTS

The author wishes to express his thanks to Dr. C. A. Tobias, Dr. R. K. Mortimer and Dr. R. A. Wijsman for valuable suggestions in connection with this paper.

SUMMARY

1. The generation times of yeast cells grown under optimum conditions are variable, but deviations from the mean generation time are not heritable.

2. The mean generation times of haploid, diploid, triploid, and tetraploid strains are nearly the same, with the exception of haploid daughter cells.

3. The generation times of a mother cell and its daughter are 100% correlated (i.e., they divide synchronously) for diploid, triploid, and tetraploid strains at 30°C. In the case of the diploid at 38° C. and the haploid at 30°C., the mother cell always divides before the daughter cell. In both cases the mother cells divide at a rate typical of the diploid at 30°C., but the daughter cells divide at a lower rate.

4. Both the mean generation time and the half-width of the generation time distribution increase at temperatures

above or below the optimum. These results are analyzed with the aid of the theory of the multiple phase birth process developed by Kendall.

5. It is proposed that the rate-limiting process in normal division is controlled by specific nongenetic units, and that the distribution of these units during the division of a cell determines the relative division times of the two daughter cells.

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CALCIUM UPTAKE IN HOMOGENIZED ORGANS FROM IMMATURE, ADULT, AND AGING RATS[1]

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THREE FIGURES

INTRODUCTION

Attempts in the past to characterize senescence in terms of chemical changes in tissues have not led to clear-cut criteria of aging. It has been established, however, that aging in animals is often accompanied by changes in the calcium content of soft tissues, as well as the skeletal (Barondes, '50; Heilbrunn, '52; Lansing, '42, '47, '52; McCay, '52; Sherman and MacLeod, '25). The longevity and fecundity of even the rotifer, in fact, has been controlled experimentally by varying the calcium content of the growth medium (Lansing, '42). While calcium usually tends to accumulate with aging, at least one author has reported a "remarkable diminution" of calcium in the muscle of a number of laboratory animals (Cahane, '27). The connection between calcium changes and aging may be fundamental because of the role of the element in cell permeability, cell division, and certain enzyme systems (Heilbrunn, '52).

Although it is recognized that blood calcium exists both in a free, diffusible form and as a "bound," non-diffusible form, evidently complexed in some way with proteins, tissue cal-

[1] This work was done in partial fulfillment of the requirements for the degree of Doctor of Philosophy and during the tenure of fellowships from the National Science foundation, the Harrison Foundation, and the Lalor Foundation.

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cium generally has been assayed without regard to such a distinction. It seems plausible that any change in the retentiveness of calcium by tissues must involve particularly the bound form, and this implies changes in cellular constituents that complex with calcium. Such constituents may include the proteins and nucleoproteins (Klotz, '46; Lansing, '47; Lansing et al., '49; McLean and Hastings, '35), lipids (Lansing et al., '49), organic sulfates (Boyd and Neuman, '51), the adenosine phosphates (Distefano and Neuman, '53), and phosphorus (Benjamin, '33).

The work reported here was designed to investigate whether changes in calcium retentiveness with advancing age could be demonstrated *in vitro*. Lansing already has shown differences in calcium-binding ability in the livers of intact mice of wide age disparity. According to this work, in which the Ca^{45} isotope was used, the livers of young mice show a low uptake and high turnover of calcium, whereas a high uptake and low turnover rate occurs in the livers of old animals. The results have been questioned (Talmadge et al., '52) on the basis of the possible introduction of non-tracer doses of carrier calcium which could have had important physiological consequences.

The experiments reported in the present paper were carried out entirely *in vitro* and the analytical techniques involved chemical methods alone. The rat, because of its conveniently short life span, for a mammal, and its easy availability, was chosen as the experimental animal. The tissues tested for calcium-binding ability were the liver, heart, brain, kidney, skeletal muscle, and skin, and these were used as homogenates or in a finely minced condition.

MATERIALS AND METHODS

At the outset it was realized that the procedures would require statistical treatment of the results in view of: (1) the likelihood that the significance of differences of small magnitude, with respect to calcium uptake, would have to be tested; (2) the necessity of sorting out individual differences from possible variation between groups; (3) the need for assessing

the extent of experimental error; and (4) the desirability of making the most effective use of the available material. On the basis of the intended statistical methods, as well as the number of operations in the individual experiments, it was determined in advance that four, widely-spaced age groups, each containing 10 animals, would be tested for calcium-binding ability of the 6 organs mentioned previously. This represented a total of 240 separate determinations, each done in duplicate. Modern statistical methods are sufficiently refined so that it is questionable whether the labor of handling larger numbers of animals would have been compensated by the resulting slight gain in accuracy.

Inbred, albino, female rats of the Wistar strain were used. With the exception of the youngest group, these had borne litters more than once, although none were gravid or lactating during the period of this study. The diet of these animals was kept uniform and consisted of dog chows, cod liver oil in diluted, condensed milk, raw pig liver, and carrots minus the greens. The health of the animals seemed uniformly good at all ages.

Calcium uptake was measured by the method of equilibrium-dialysis (Klotz, '46). The entire heart, both kidneys, and portions of the psoas muscles and of the liver of each rat were blended for 30 seconds, by means of a Virtis homogenizer, in cold 0.005 M $CaCl_2$ solution (0.20 mg Ca/ml) buffered to pH 8.0 with 0.05 M "tris" buffer (trishydroxymethylaminomethane). The entire brain was similarly treated, except that a glass Potter-Elvehjem type of homogenizer was used to minimize foaming. A portion of skin, stripped by means of a razor blade of the hair and adhering muscle and fascia, was minced as finely as possible with scissors and likewise suspended in the cold, buffered $CaCl_2$ solution. It must be mentioned here that any variation in the data, especially in comparing skin with the other organs, cannot be attributed to the state of division of the prepared tissues. A separate series of experiments, in which the tissues were graded from coarsely chopped preparations to homogenized suspensions, indicated no differ-

ence in calcium uptake at the same ratio of tissue weight to calcium concentration. All suspended tissues were placed into cellophane sacs and immersed in equal volumes of the same $CaCl_2$ solution, then stored overnight in a refrigerator. Diffusion equilibrium apparently was completed in that period so that the extent of maceration was immaterial. The original purposes of homogenization were easier handling and assurance of intimate contact of the medium with the cellular contents. Shaking of the suspensions likewise was found to be dispensible.

In the rationale of equilibrium-dialysis, the calcium concentration of the circumambient solution will drop as calcium moves into the sac to reestablish the equilibrium that was disturbed upon the removal of calcium by the tissue. The reduction in outside concentration is taken as the measure of calcium-binding by the homogenized tissue. To each of 5 ml aliquots of the solution outside the cellophane membranes was added 1 ml of 1 N NaOH and 3 drops of the indicator, murexide (75 mg dissolved in 50 ml of absolute ethylene glycol; see Brunisholz et al., '53). This solution then was titrated with a 0.01 M solution of the disodium salt of ethylenediaminetetraacetic acid (EDTA). One milliliter of this EDTA solution was equivalent to 0.40 mg of calcium. The endpoint was taken as the first sharp change from the reddish to the bluish color (Greenblatt and Hartman, '51).

It was found that the amount of calcium removed from a solution by animal tissue will increase slightly, and more-or-less linearly, as the concentration of either component is increased in the homogenate. This parallels the situation found in the complexing of calcium by casein (Greenberg et al., '35). In the present case, the ratio of 0.1 gm of tissue (wet weight) to each milliliter of a solution containing 0.20 mg Ca/ml (0.005 molar) was selected arbitrarily on the basis of the amount of tissue available for a particular experiment, the sensitivity of the analytical method, and the relatively minute absolute quantities of calcium that are taken up by the tissue.

The large number of methods reported in the literature for micro and semi-micro determination of calcium attest to the difficulties of accurate measurement. In the present case the question was begged by measuring changes in concentration, rather than absolute quantities. These then were translated arithmetically to milligrams of calcium uptake per gram of wet weight or dry weight of tissue. Dry weights of tissue were determined from samples of the excised tissues placed in an oven at 100°–110°C. until constant weight was attained.

All individual experiments were done with the organs of a single animal, except in the youngest group, in which the organs of three animals were pooled and sampled in order to obtain sufficient tissue. The statistical calculations are based upon the means of duplicate determinations.

A blank was used with each set of sacs, consisting of equal volumes of the buffered $CaCl_2$ solution inside and outside the permeable membrane. A series of controls were run at one time for the entire range of experiments to determine how much calcium disappearance could be attributed to adsorption onto more-or-less finely divided or partially soluble materials that presumably were chemically inert to calcium ions. The substances tested were suspensions of soluble starch, shredded cellulose, and unmelted agar. A small, but fairly constant, drop in calcium concentration (5–10%) actually occurred in all solutions in contact with these materials. Although adsorption may or may not have been the sole factor involved, this was assumed as a constant error, when tissues were used, and eliminated from further consideration, since only relative activities were sought.

RESULTS

Table 1 summarizes the raw data as the mean values of 10 determinations for each organ (in duplicate) in each age group. Group I corresponds to rats aged 30 to 37 days; group II rats were 5 to 5½ months old; group III rats were aged 14 to 15 months; and group IV rats were 26 to 28 months of age. Although comparisons can be rough only, it is gener-

ally agreed that such rats would correspond to about 3 year old, 15 to 16 year old, 42 to 45 year old, and 78 to 84 year old persons, respectively.

Tables 2 and 3 present the mean determinations in terms of milligram Ca uptake per gram of tissue, wet weight and dry weight, respectively. While the dry weight determinations are more indirect than the wet weight, the elimination of water content from the calculations has the effect of approaching the common denominator of all the determinations, the solid phase, which is directly involved in the chemical interactions. This procedure tends also, of course, to eliminate variation in

TABLE 1

Average uptake of calcium by homogenized rat organs from a solution containing 0.20 mg Ca/ml Solution

(in mg Ca/ml ± standard error)

AGE:	GROUP I	GROUP II	GROUP III	GROUP IV
Organ:				
Heart	.112 ± .004	.123 ± .002	.109 ± .004	.111 ± .004
Muscle	.130 ± .002	.109 ± .003	.109 ± .002	.110 ± .003
Kidney	.129 ± .002	.124 ± .002	.111 ± .003	.111 ± .003
Brain	.098 ± .002	.099 ± .002	.092 ± .002	.104 ± .001
Liver	.113 ± .002	.110 ± .002	.106 ± .002	.110 ± .003
Skin	.081 ± .003	.038 ± .001	.032 ± .002	.039 ± .003

the weight measurements arising from differences in the "wetness" of the tissues.

In both tables, 2 and 3, it is evident that there is little obvious difference among the figures, with the exception of those for skin when contrasted with the other organs. It is necessary, therefore, to use statistical tools to test for the possibility of real differences. The summations shown in tables 2 and 3 are used in each analysis of variance, according to the two criteria of age and organ, that follows its appropriate table.

Each analysis shows a low probability (P) that the differences in calcium uptake, both among the various organs and among the 4 age groups, are due merely to chance. Stated

positively, the low P values indicate that the differences are significant. Because skin shows such distinctive differences from all the other tissues, however, it seems possible that it alone contributes heavily to the calculated probabilities. As a matter of fact, if the analyses of variance are recapitulated,

TABLE 2

Calcium uptake by rat organs
(mg Ca/gm tissue wet weight ± standard error)

AGE:	GROUP I	GROUP II	GROUP III	GROUP IV	TOTAL
Organ:					
Heart	1.124 ± .027	1.226 ± .024	1.071 ± .021	1.156 ± .085	4.577
Muscle	1.305 ± .019	1.090 ± .024	1.091 ± .017	1.102 ± .035	4.588
Kidney	1.295 ± .023	1.238 ± .022	1.099 ± .027	1.109 ± .029	4.741
Brain	0.971 ± .020	0.979 ± .015	0.908 ± .015	1.006 ± .024	3.864
Liver	1.124 ± .019	1.092 ± .023	1.074 ± .021	1.087 ± .023	4.377
Skin	0.801 ± .030	0.381 ± .012	0.341 ± .024	0.390 ± .027	1.913
Total	6.620	6.006	5.584	5.850	24.060

$N = 24$ $\quad \Sigma X = 24.060$ $\quad \Sigma X^2 = 25.776800$

$$\text{Correction term} = \frac{(\Sigma X)^2}{N} = 24.120150$$

$$\text{Total sum of squares} = \Sigma X^2 - \frac{(\Sigma X)^2}{N} = 1.656650$$

$$\text{Among columns S.S.} = \frac{\Sigma(\text{Col. totals})^2}{N_C} - \frac{(\Sigma X)^2}{N} = 0.096515$$

$$\text{Among rows S.S.} = \frac{\Sigma(\text{Row totals})^2}{N_R} - \frac{(\Sigma X)^2}{N} = 1.435835$$

(N = total no. of items; N_C = no. of items in a column; N_R = no. of items in a row)

$$\text{Degrees of freedom} = \text{D.F.} = \underset{\text{(Tot.)}}{N-1} = \underset{\text{(among col.)}}{N_R-1} + \underset{\text{(among rows)}}{N_C-1} + \underset{\text{(remainder)}}{(N_C-1)\,(N_R-1)}$$

	S.S.	D.F.	MEAN SQUARE	F	P
Among columns (ages)	.096515	3	.0321716	3.88	$< .05 > .01$
Among rows (organs)	1.435835	5	.2871670	34.65	$< .001$
Remainder (error)	.124300	15	.0082866	....	
Total	1.656650	23			

but this time with the omission of the figures for skin, the differences between the age groups in table 2 alone are found to be no longer significant ($.05 < P < .20$). The previous conclusions are unchanged, however, as far as table 3 is concerned and the differences between organs in table 2.

TABLE 3

Calcium uptake by rat organs
(mg Ca/gm tissue dry weight ± standard error)

	GROUP I	GROUP II	GROUP III	GROUP IV	TOTAL
Organ:					
Heart	5.06 ± .11	5.03 ± .12	4.59 ± .13	4.66 ± .21	19.34
Muscle	5.80 ± .15	4.38 ± .11	4.17 ± .12	4.24 ± .18	18.59
Kidney	5.39 ± .06	5.39 ± .08	5.12 ± .12	5.34 ± .14	21.24
Brain	5.04 ± .12	4.71 ± .10	4.36 ± .07	4.84 ± .14	18.95
Liver	3.95 ± .08	3.53 ± .07	3.56 ± .12	3.57 ± .06	14.61
Skin	2.51 ± .11	0.94 ± .04	0.75 ± .06	0.94 ± .05	5.14
Total	27.75	23.98	22.55	23.59	97.87

$N = 24$ $\Sigma X = 97.87$ $\Sigma X^2 = 446.8459$

Correction term = 399.1057
Total sum of squares = 47.7402
Among columns S.S. = 2.5766
Among rows S.S. = 43.3281

	S.S.	D.F.	MEAN SQUARE	F	P
Among columns (ages)	2.5766	3	.8588667	7.01	$< .01 > .001$
Among rows (organs)	43.3281	5	8.6656200	70.82	$< .001$
Remainder (error)	1.8355	15	.1223667		
Total	47.7402	23			

In view of the above, the question of age differences in table 2 must be looked into a little further. The fact that the analysis of variance is based upon the *means* of the actual determinations, rather than directly upon the raw data, may tend to obscure the real differences between the age groups. Calculation of the confidence intervals, on the other hand, based upon the individual observations rather than the means, indicates that some age groups actually are significantly different from

others with respect to the behavior of particular tissues. Figures 1 and 2 present the graphic trends of calcium uptake with age for the various tissues and show the confidence intervals calculated from the wet weight and dry weight data. In all cases where there is little or no overlap of the confidence intervals about the plotted points, the differences are signifi-

TABLE 4

Water content of rat tissues
(Per cent)

AGE:	GROUP I	GROUP II	GROUP III	GROUP IV	TOTAL
Organ:					
Heart	77.8	75.6	76.3	76.6	306.3
Muscle	77.4	75.0	73.3	73.6	299.3
Kidney	75.9	76.8	78.3	79.1	310.1
Brain	80.7	79.1	79.1	79.0	317.9
Liver	71.1	69.1	69.8	69.8	279.8
Skin	67.9	59.2	57.8	58.8	243.7
Total	450.8	434.8	434.6	436.9	1757.1

$N = 24$ $\Sigma X = 1757.1$ $\Sigma X^2 = 129673.35$

$$\text{Correction term} = \frac{(\Sigma X)^2}{N} = 128641.68.$$

$$\text{Total sum of squares} = \Sigma X^2 - \frac{(\Sigma X)^2}{N} = 1031.67.$$

$$\text{Between groups S.S.} = \frac{\Sigma(\text{Class totals})^2}{N_c} - \frac{(\Sigma X)^2}{N} = 30.06.$$

$$\text{Within groups S.S.} = \Sigma X^2 - \frac{\Sigma(\text{Class totals})^2}{N_c} = 1001.61.$$

$$\text{Degrees of freedom} = \text{D.F.} = \underset{\text{(tot.)}}{N-1} = \underset{\text{(within)}}{N-k} + \underset{\text{(among)}}{k-1}$$

(N = total no. of items; N_c = no. of items in a class; k = no. of classes; s'^2 = estimated variance.)

	S.S.	D.F.	s'^2
Between	30.06	3	10.02
Within	1001.61	20	50.03
Total	1031.67	23	

$$F_2^{20} = \frac{50.03}{10.02} = 4.99 \qquad .05 < P < .20$$

cant, and this conclusion has been doubly assured by the application of t-tests to particular pairs of values (the t-tests are not presented here for the sake of brevity).

The findings can be summarized as follows:

1. With respect to heart, figure 1 shows a higher uptake in groups II than in groups I and III, but not group IV. In figure 2, however, the relative positions of groups I and II are inter-

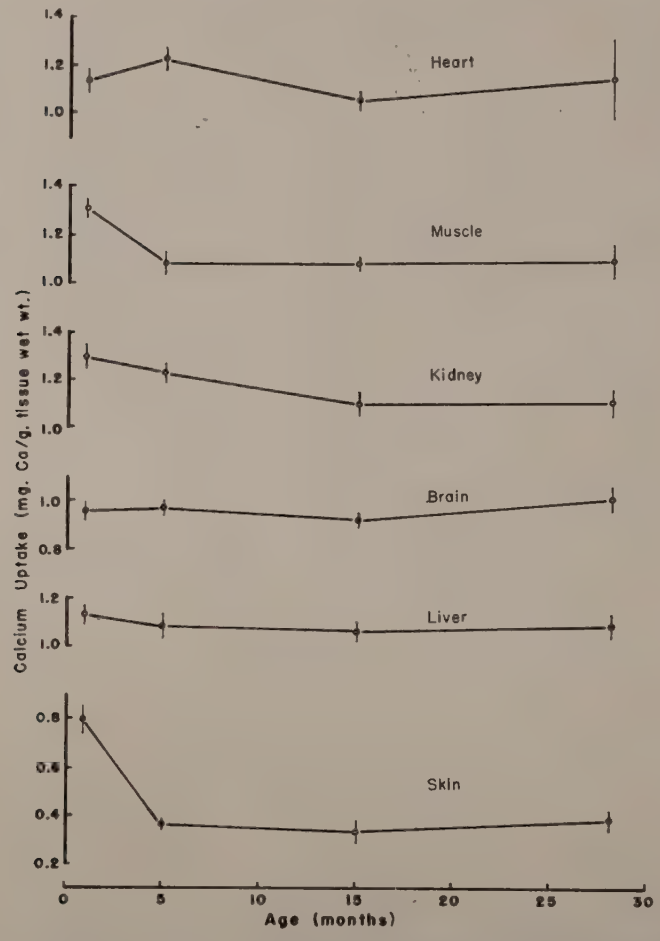

Fig. 1 Ninety-five per cent confidence intervals of determinations of mean calcium uptake (wet weight basis).

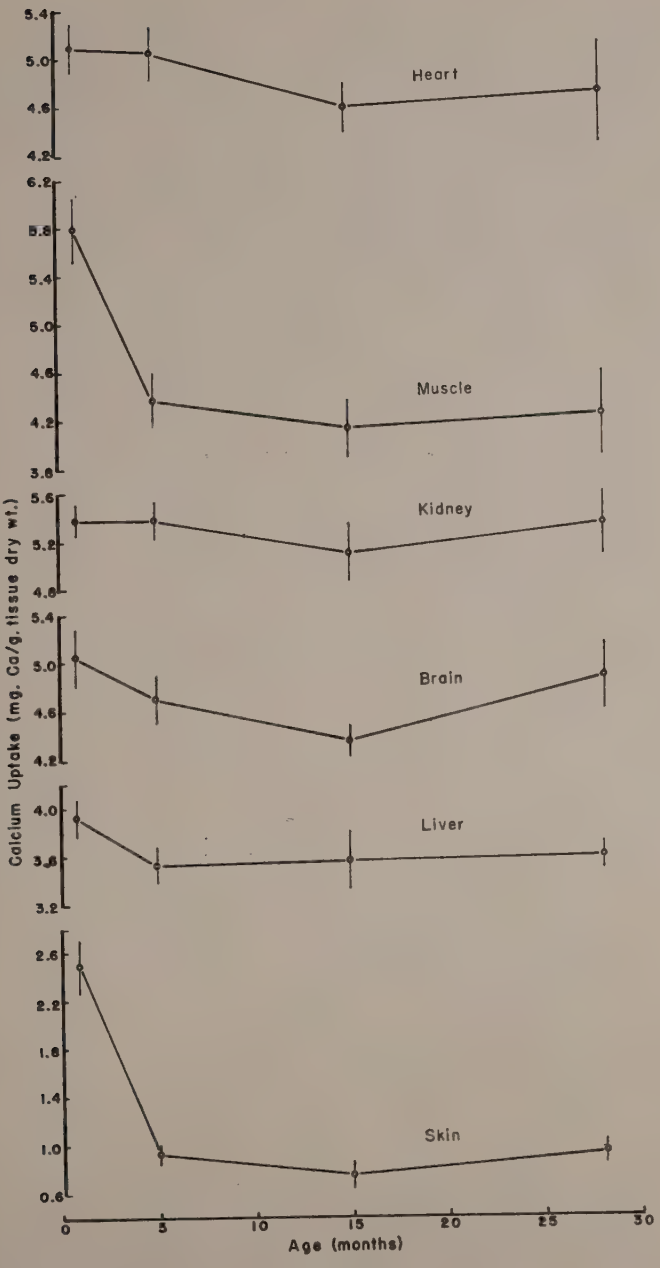

Fig. 2 Ninety-five per cent confidence intervals (dry weight basis).

changed, but the trend remains essentially unchanged as an early high followed by a lower uptake in later life.

2. In the case of muscle, both figures show a significantly higher uptake in group I than in all the other age groups.

3. For kidney, figure 1 shows a significantly higher uptake in groups I than in groups III and IV, with group II intermediate. While the general form of the corresponding curve in figure 2 is similar, the significance of the differences here is unproven.

4. Calcium uptake by the homogenized brain falls to a minimum in group III in both figures.

5. On a wet weight basis, there is no significant change with age in the case of liver. The dry weight curve, however, shows a maximum in group I.

6. Calcium uptake is substantially higher in the skin of group I rats, while the dry weight curve shows, in addition, a significant minimum in group III.

7. In summary, the overall impression is that of a trend from a higher reactivity of the tissues with calcium early in life to a more-or-less uniform low from young adulthood to old age.

Water content of the tissues. The possibility was tested and eliminated that any differences in calcium uptake are a reflection of changes in water content with age. Table 4 represents the means of 10 determinations of per cent water content of each tissue in each age group. The ranges and standard errors are not shown, but they are extremely small. These figures indicate that the hydration of each kind of tissue is fairly characteristic and changes little throughout life, in opposition to the report of Donaldson ('24). For the sake of greater assurance on the latter point, however, an analysis of variance, this time with the single criterion of age, is presented with the table. The high value of P indicates that the differences in water content with age, observed in these experiments, are not significant and could have occurred by chance alone. The high hydration of the skin of baby rats is the exception.

While no attempt was made in the present work to identify the calcium-binding cellular entities, it would be of interest to know the degree if selectivity of the calcium ions in their interaction with the constituents of the disrupted tissues. It is likely that proteins are chiefly implicated so that the problem then would be to characterize the proteins involved. Unfortunately, preliminary investigations of the supernatant solutions of the homogenates in this case indicated that electro-

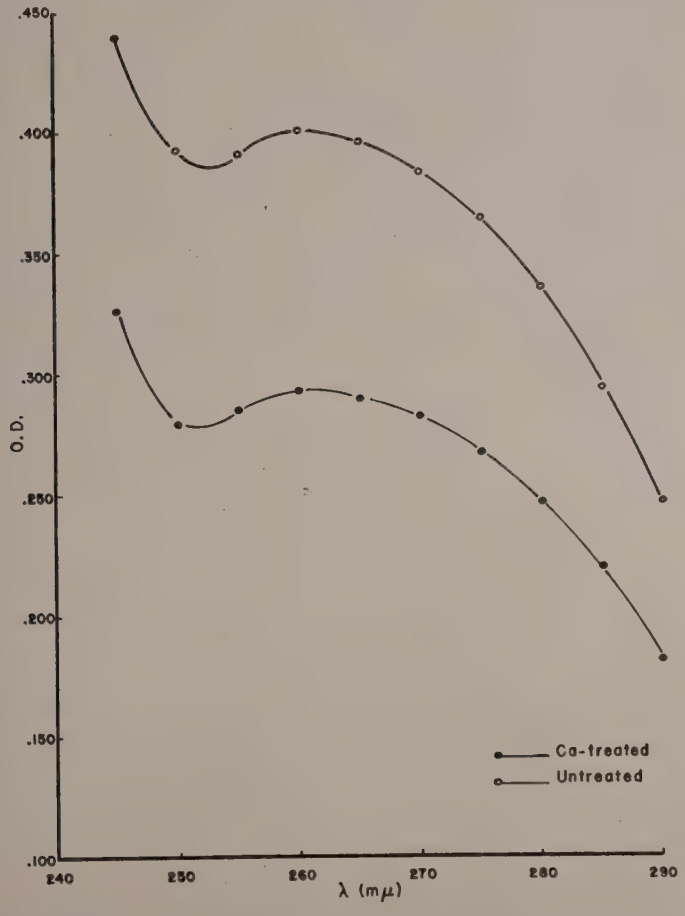

Fig. 3 Ultra-violet absorption by Ca-treated and untreated supernatant solutions of rat liver homogenate (100 × dil.).

phoretic patterns could not be interpreted adequately until extensive fractionation of the salt extracts is first accomplished. This was reserved for later investigation. It is possible, nevertheless, to show that the calcium taken up by the tissue enters into an insoluble complex of some sort. Figure 3 compares the ultraviolet absorption spectra of the supernatant solutions of liver homogenates that had been prepared in a buffered $CaCl_2$ solution in one case and in the buffer without calcium in the other. The solution strengths are as before. The maxima present in each curve correspond to nucleic acid absorption at 260 mμ, but are much reduced in prominence because of interference from the proteins present. Addition of calcium to the supernatant solution displaces the curve downward, owing to the removal of solid material, without essentially changing its form. Calculations using the "280/260 ratio" (Warburg and Christian, '41) indicated an estimated drop of about 39% in the protein content of the supernate in this particular experiment, and a nitrogen analysis by the Nessler method showed the same precentage decrease in total nitrogen when calcium was added.

DISCUSSION

The statistics have already been discussed in conjunction with the particular data concerned. To recapitulate, the experiments indicate the following points:

1. Each of the tissues tested has shown a fairly characteristic reactivity with calcium ions at the particular concentrations of tissue and salt used here. This qualification is necessary because the chemical interaction of tissues (or proteins) and calcium is not stoichiometric and depends upon concentration (Klotz, '46). The principal source of error in all these experiments lay in the necessity of dealing with concentration changes, rather than directly with absolute quantities of calcium, and the need for arithmetical inference of the latter from the concentration measurements.

2. Under the conditions defined by the work, it is possible to show significant differences between rats of various ages in

the way that their tissues react to added calcium *in vitro*. With a few exceptions, the trend seems to proceed from a high initial activity of the tissues to a general low beginning somewhere between the first and fifth month of life in the rat. The absolute quantities involved are very small, but the order of magnitude is the same as that of the actual concentrations of calcium found in the soft organs of mammals. It is interesting that a similar pattern of change with age was found by Cahane ('27) in the calcium content of the organs of several other laboratory animals.

3. The addition of calcium ions to a homogenate seems to result in the removal of protein from the soluble fraction. The UV absorption curve of the supernatant solution is displaced downwards, without any essential change in form, indicating that the nucleic acids are affected as well as the proteins. Indeed, Neuberg and Roberts ('49) have proved the powerful calcium-binding capacity of nucleic acids and nucleotides by using them to solubilize water insoluble calcium phosphate. An insoluble complex is formed when the amount of calcium present is excessive, relative to the nucleic acid. It is likely that in the present case, the addition of calcium results in the formation of an insoluble Ca-nucleic acid-protein complex. In systems as crude, from a biochemical standpoint, as these homogenates it does not seem possible to detect any special selectivity in the effects of the calcium ion. If it should ever be found that calcium is associated with different cellular components as animals age, then the implications for senescence studies would be highly important.

4. The total water content of the various rat tissues remains unchanged to any important degree throughout the life of the rat, except for the high degree of hydration of the skin in the very young animal. Donaldson ('24), on the contrary, has claimed a gradual loss of water in all rat organs with advancing age and, indeed, it seems to be a popular conception that dehydration accompanies senescence in most animals.

5. Since water content is constant with age and calcium uptake varies, there is obviously no significant degree of cor-

relation between these two factors, although one might have been expected. Brain, for example, has a consistently lower uptake, while being the most hydrated of the tissues used. On the other hand, the period of highest uptake by the skin corresponds with the time of highest water content of that tissue.

The overall conclusion from this research seems inescapable that calcium is not implicated in senescence under the terms of the experimental procedures. Any differences in the various homogenized organs, relative to calcium uptake, occur primarily between the postnatal period and adulthood. This fact and the data of Cahane ('27) suggest that calcium uptake and retention are correlated, if anything, with active growth rather than senescence. If calcium occurs in greater quantities in youthful animals, the present work suggests that this is so because of differences in inherent chemical reactivity in the tissues and cells of young and old animals. Age differences of this sort, of course, are found on the molecular level and have nothing to do with structural changes in the cell or with alterations in the cellular environment *per se*.

Of most immediate interest to these conclusions are the results obtained by Lansing with mice, referred to previously ('49), which led to the opposite viewpoint. It should be noted that there are two important differences in approach in the two experiments. For one thing, mice were used in the one case and rats in the other. It is possible, even probable, that senescence is not the identical process or processes in all organisms, so that observations in one kind of animal cannot be generalized safely to even closely related species. In the second place, Lansing and his coworkers studied calcium uptake in the livers of intact animals, while in the present work all tissues were first excised and then disrupted in a calcium solution. Aside from the criticism of Talmadge et al. (referred to earlier, '52), it is pertinent to the question that the liver is a homeostatic organ of prime importance in the living animal. Lansing's results, therefore, may have reflected the reaction of the liver to a temporary hypercalcemia, thus illustrating a

change with age in response to stress, rather than the direct implication of calcium in senescence.

An obvious defect in the work reported in this paper is the use of tissues that had not been decalcified before their exposure to calcium. The fact that these tissues, already in equilibrium with calcium in their normal environment, were able to take up additional amounts at all is attributable to the operation of the mass action law of these reactions, assuming proteins as being principally involved (Klotz, '46; McLean and Hastings, '35). As has been stated already, the more calcium that is presented to a given amount of tissue (or protein, in Klotz's experiments, '46), the more will be taken up. It is unfortunate that any attempt at prior decalcification probably must alter the unstable tissue components, especially the proteins, in an unpredictable way. In this way access would be blocked to one of the central problems of this study: Do animal tissues complex calcium differentially with aging and, if so, does this reflect some subtle chemical change in cellular composition that is undetectable by the usual methods of analysis? Nevertheless, the task of any continuation of this research must be to determine whether or not all tissues started from about the same point on the uptake *vs.* concentration curve or whether, for that matter, most tissues have the same or different uptake curves.

SUMMARY AND CONCLUSIONS

1. Rat tissues, such as heart, psoas muscle, kidney, brain, liver, and skin, when homogenized or otherwise comminuted in a given concentration of calcium chloride solution, will take up an amount of calcium which is characteristic for each tissue.

2. Of four age groups tested — approximately 1 month, 5 months, 15 months, and 28 months of age — the mean uptake of calcium by any particular tissue usually was significantly different in one or two of the age groups. In most cases the change in reactivity towards calcium occurs between the ages

of 1 month and 5 months and calcium uptake, where such change occurs, is highest in early life.

3. Water content of the organs does not vary significantly throughout the life span covered by these experiments. No significant correlation was observed between calcium uptake by a tissue and its water content.

4. Spectrophotometric and chemical analysis of the supernatant solutions of liver homogenates, exposed in one case to calcium and not in the other, indicate that calcium reduces the solubility of certain components in the homogenate. The components were not identified, but proteins and nucleic acids are evidently affected.

5. It is concluded that these *in vitro* experiments do not show a significant role for calcium in the senescence of rats. Rather, the observed changes suggest that maximal calcium affinity is associated more closely with the processes of active growth, possibly reflecting a different chemical composition in young tissues.

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THE ROLE OF PHOTOSYNTHESIS IN THE PHYSIOLOGY OF OCHROMONAS [1]

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FOUR FIGURES

Among the algae and related protists there are found the nutritional extremes of both autotrophy and heterotrophy. *Chlamydomonas moewusii* (Lewin, '50) and several of the blue-green algae (Kratz, '54) are obligate autotrophs in the sense that they will utilize for growth no carbon source other than carbon dioxide. At the other extreme there are colorless species such as Prototheca (Barker, '36) and the induced and permanently apochlorotic forms of Euglena (Hutner and Provasoli, '51) which are limited to a heterotrophic nutrition. Many of the algae which have been studied may grow either autotrophically or heterotrophically although in Chlorella and Scenedesmus, and probably rather commonly, photosynthesis will support a rate of growth higher than can be attained on any organic substrate in the dark (Myers, '51). From such extreme and intermediate cases one is led to expect a complete spectrum of relationships between autotrophic and heterotrophic nutrition within the algae. Of particular interest in this connection is Ochromonas.

The chrysomonad *Ochromonas malhamensis* was isolated by Chen and described by Pringsheim ('52) as a nutritionally versatile organism which is saphrotrophic, phagotrophic, and phototrophic. It is a flagellate protozoan considered also an alga by virtue of its brownish-green pigmentation. A defined medium for the Pringsheim strain and several other isolates

[1] Aided by Grant G259 from the National Science Foundation.

has been worked out by Hutner, Provasoli, and Filfus ('53). The carbon source may be supplied as glucose or sucrose or as particulate material such as starch or oil droplets. Evidence for photosynthesis has been based only on the observation of Pringsheim that illuminated cultures without an organic carbon source would remain viable, though without multiplication, for long periods. The role of photosynthesis in the nutrition of Ochromonas is the subject of the present investigation.

METHODS

A culture of *Ochromonas malhamensis* was provided by the kindness of L. Provasoli; this is the same strain used by Pringsheim ('52), Hutner et al. ('53), Ford ('53) and Reazin ('54). The medium recommended by Hutner et al. and two modifications used are presented in table 1. Medium H is taken directly from Hutner et al.; media S and G are modifications designed to restrict to a minimum utilizable organic materials.

Cultures were grown in 22 × 180 mm or 38 × 300 mm test tubes immersed in water baths, aerated with 4% CO_2 in air, and in darkness or illuminated on each side by daylight fluorescent lamps at about 500 foot-candles (Myers, '50). The larger tubes were used to produce cell material for manometric experiments; the smaller tubes were used for growth rate studies by readings of optical density (D) in an Evelyn colorimeter with 600 mμ filter. Growth rates are reported in terms of the specific growth rate, $k = \frac{1}{t} \log_{10} \frac{D}{D_o}$, in the convenient units of $\log_{10}$ units per day; when k is 0.302, the generation time is one day. All experiments reported herein were at 25 ± 0.1°C.

Rates of photosynthesis were measured by the indirect method of Warburg using as suspending fluid the growth medium saturated with 5% CO_2. Illumination was provided by a bank of tungsten lamps underneath the bath or by a grid of 4 closely-spaced neon discharge tubes held in the bath just below the reaction vessels and operated at 60 or

120 milliamps. Respiration was studied by both the direct and indirect methods. Because of variable behavior, apparently resulting from centrifuging, all experiments reported below used cells transferred directly or with dilution from cultures to manometric vessels. Dry weight, as a measure

TABLE 1

Media for Ochromonas

Quantities per liter; final pH 4.9–5.3

	H[1]	S	G[2]
KH_2PO_4	0.3 gm	0.3 gm	0.3 gm
$MgCO_3$ (basic)	0.5 gm	...	...
$MgSO_4 \cdot 7H_2O$	...	1.5 gm	0.6 gm
$CaCO_3$	0.15 gm	...	...
$CaCl_2$	...	0.17 gm	0.17 gm
NH_4HCO_3	0.4 gm	...	...
$(NH_4)_2SO_4$	...	...	2.5 gm
$(NH_4)_2H$ citrate	1.2 gm	...	...
EDTA[3]	...	...	0.5 gm
Trace Metal mix no. 2[4]	10.0 ml	...	...
Trace Metal mix no. 42[5]	...	...	12.0 ml
Mo (as NH_4 salt)	20 mg	...	20 mg
Thiamin·HCl	2 mg	2 mg	2 mg
Biotin	4 μg	4 μg	4 μg
Cobalamin	5 μg	5 μg	5 μg
L-Histidine·HCl·H_2O	0.5 gm	...	0.5 gm
L-Glutamic acid	3.0 gm	...	...
DL-Methionine	0.6 gm	...	...
L-Arginine·HCl	0.4 gm	...	...
Sucrose	12.0 gm	...	...

[1] Based on table 1 of Hutner et al. ('53).

[2] Based on Hutner et al., p. 857.

[3] Ethylenediamine tetraacetic acid.

[4] "Chrysomonad Metals no. 2" of Hutner et al. providing Zn, Mn, Fe, Cu, Co.

[5] "Metals no. 42" of Hutner et al. providing Zn, Mn, Fe, Cu, Co, B.

of cellular material, was determined on separate aliquots of original suspension by centrifuging at 1400 RCF, pouring off the supernatant medium, transferring the cells in small volumes of water to tared crucibles, and drying at 90–100°C. Small errors due to occluded medium were preferred to the larger errors of cell lysis if the cells were washed in water.

Balance studies of metabolism followed the essential features of the method of Myers and Johnston ('49). The vessel was a 2-liter Erlenmeyer flask containing about 200 ml of cell suspension, shaken slowly in a Warburg bath in darkness or over a grid of daylight fluorescent tubes at an illumination of about 200 foot-candles. Carbon dioxide and oxygen concentrations in the gas phase were obtained by Haldane analyses to 0.01% at the beginning and end of the experiment. Glucose was determined by the Shaffer-Somogyi method (Somogyi, '52).

RESULTS

1. Growth studies. Comparative rates of growth in light and in darkness are illustrated in figure 1 as observed in medium H. When glucose or sucrose are provided as the carbon source almost equal rates are observed in light and in darkness. The higher rate is 0.5 $\log_{10}$ units per day, equivalent to a generation time of about 14 hours. This is comparable to the growth rates of several of the Euglenas (Cramer and Myers, '52) and a little better than one-half of that of *Chlorella pyrenoidosa.* With glycerol the growth rate is significantly higher in light. With only carbon dioxide provided there is an approach to a very low growth rate in light but rapid deterioration in the dark.

Figure 2 presents an experiment on the effect of light under anaerobic conditions. Cultures growing in medium H + sucrose + light + 4% CO_2 in air were transferred to 4% CO_2 in nitrogen in light or in darkness. No growth is supported by anaerobic darkness although the cells remain viable; in light growth is resumed after transfer and continues at a close approach to the rate of a control culture under 4% CO_2 in air. The experiment has been repeated several times. Cultures aerated with 4% CO_2 in nitrogen, or with tank nitrogen alone which gives almost equivalent results, may be carried through several transfers without decrease in growth rate. Clearly the organism has either a normal photo-

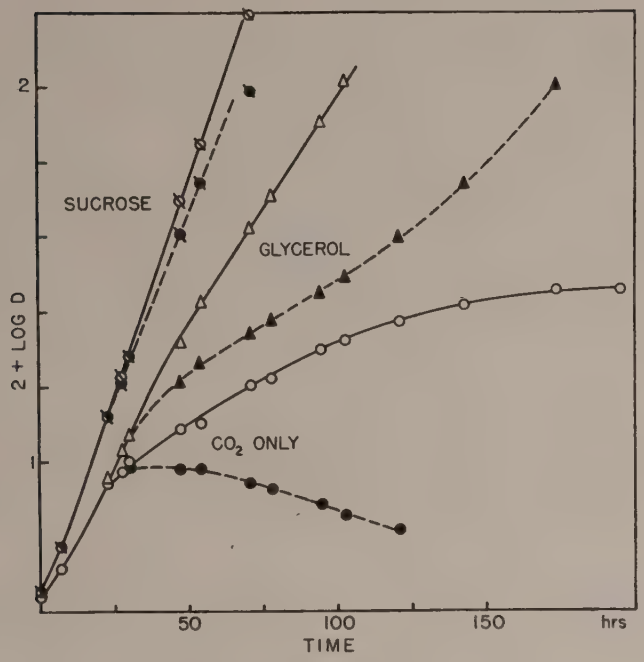

Fig. 1 Growth of Ochromonas in medium H with 4% CO_2 and with 4% CO_2 plus sucrose or glycerol. In darkness, solid points, broken lines; in light, open points, continuous lines.

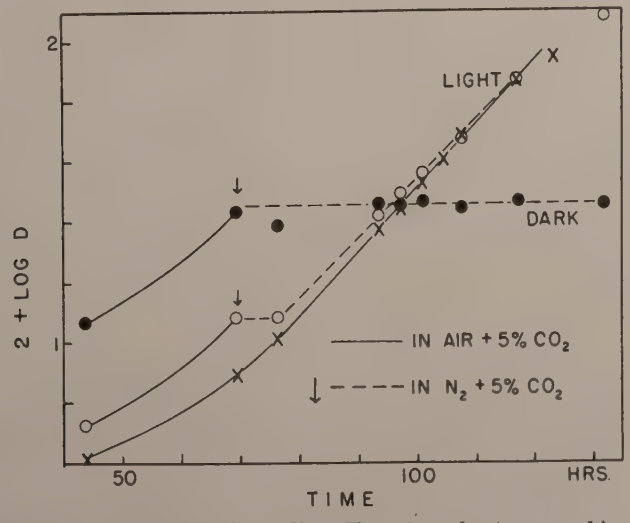

Fig. 2 Growth of Ochromonas in medium H on transfer to anaerobic conditions.

synthesis, which maintains an internal aerobic condition in the cells, or some form of a photoreduction mechanism.

In order to allow further test of the ability to support growth by photosynthesis alone, efforts were made to further simplify the medium to a minimum of utilizable organics. Cells grown in medium H and transferred to starvation medium S were found to just about maintain their weight over a two or 4 day period in the light as compared to a rapid loss in weight (— 75% in 4 days) in the dark. However, medium S was considered inadequate for long-time growth

TABLE 2

Effects of deletion of amino acids

In medium G + 1% sucrose; aeration with 4% CO_2 in air; amino acid concentrations equivalent to those shown for medium H, table 1; values cited are for specific growth rate k in $\log_{10}$ units/day observed in the second and third serial subcultures.

	EXP.	− HISTIDINE	+ HISTIDINE	+ { HISTIDINE, ARGININE, GLUTAMIC, METHIONINE
Dark	1	...	0.22	0.35
	2	0.24	0.26	...
Light	1	...	0.36	0.42
	2	0.36	0.40	...
	3	0.39	0.39	...

experiments because of lack of a nitrogen source and trace elements. Medium G, with added ammonium ion and utilizing EDTA instead of citrate as the chelating agent, was examined. Table 2 represents the data of several experiments on effects of deletion of amino acids considered as possible requirements from the work of Hutner et al. ('53). Growth was always somewhat slower in medium G than in medium H. In the dark there is a clear effect of deletion of the three amino acids arginine, glutamic acid, and methionine; in the light all three amino acids may be deleted without significant effect. No evidence could be found for an expected requirement for histidine.

Further experiments in medium G without sucrose and with or without histidine demonstrated a continuing growth through three serial transfers with a total multiplication of 550 times. The very low growth rate, estimated at 0.09 to 0.11 $\log_{10}$ units per day, corresponds to a generation time of about three days. The longest of the experiments, terminated at 27 days after 9 generations, is the best evidence obtainable that Ochromonas can support continuing growth by photosynthesis with carbon dioxide as the only carbon source.

TABLE 3

Results of typical balance experiments

	MEDIUM G					
	+ GLUCOSE		+ GLUCOSE		− GLUCOSE	
	Dark Experiment no.		Light Experiment no.		Light Experiment no.	
	45	49	48	50	55	56
Duration, hrs.	96	95	74	72	41	66
Initial CO_2, %	0.03	0.03	2.55	0.03	4.81	4.82
Initial cells, mg	2.5	2.2	5.3	4.3	110	110
ΔCO_2, ml_{STP}	93.9	68.4	84.7	85.9	— 34.9	— 61.3
ΔO_2, ml_{STP}	— 83.3	— 71.9	— 69.2	— 69.8	36.6	64.2
Δglucose, mg	— 314	— 182	— 199	— 189	. . .	. . .
Δcells, mg	114	66.3	87.7	72.8	27.2	60.4
Carbon recovery, % [2]	85	96	110	109	73	92
mg glucose/mg cells	— 2.8	— 2.8	— 2.3	— 2.6	. .	. .
ml CO_2/mg cells	0.83	1.03	0.97	1.18	— 1.28	— 1.01
$\Delta CO_2/\Delta O_2$	1.13	0.95	1.22	1.23	0.95	0.95
k, $\log_{10}$ units/day	0.42	0.38	0.41	0.42	0.06	0.07

[1] Cells grown in medium H or medium G + sucrose, transferred to medium G, and incubated in light with 4% CO_2 aeration for one day before the beginning of an experiment.

[2] Calculated on assumption that all cell carbon came from glucose or CO_2; based on an estimate of 48% carbon in the harvested cells.

2. Balance studies. Large batch cultures were used to obtain balances between cells produced, glucose utilized, and the gas exchange. Table 3 presents the results of 6 representative experiments out of a total of 12 conducted in medium G. The data are not as consistent as those of similar studies with other algae. The data of lowest precision are those for dry weight of the cells produced.

In 0.5% glucose light has little effect on the over-all character of metabolism except for a small but probably significant increase in the CO_2/O_2 quotient. Any large contribution of photosynthesis to metabolism would have caused a marked change in the ratios mg glucose/mg cells and ml CO_2/mg cells. When cells are grown in light plus glucose and then transferred to medium G they lose weight during the first 24 hours; at 48 hours they have about regained their original weight; thereafter they show continued gain in weight accompanied by carbon dioxide uptake and oxygen evolution. Typical data are shown by the last two columns of table 3. Again there is evidence that a slow cell synthesis may be supported by photosynthesis alone. Rates of cell synthesis, calculated in terms of specific growth rate from initial and final cell dry weights, show reasonable agreement with values based upon optical density readings in experiments of longer duration (table 2).

3. Manometric studies. Cells growing with glucose or sucrose show a respiratory rate greater than that of other algae. Light, even at light-saturating intensity, never gives any positive O_2-evolution but only a partial suppression of O_2-uptake (table 4 A). When cells are starved in the dark in medium S the respiratory rate is decreased, but there is also a loss of pigment and decreased photosynthesis. Reazin ('54) has demonstrated that such dark-starved cells become very sluggish in their response to added carbohydrate substrate, although they respond more readily to acetate or palmitate. When cells are starved in the light, i.e., provided with no carbon source except carbon dioxide and incubated in light, they show a decreased rate of endogenous respiration

but retain ability to respire carbohydrate (Reazin, '54). Light-starved cells have been examined in some detail.

In dark experiments various substrates were added at 1% concentration to cell preparations obtained by incubating cells for a day in medium S. Glucose addition gave a three- to four-fold increase in rate. A two- to three-fold increase resulted from addition of sucrose, triacetin, glycerol, and

TABLE 4

Manometric observations of gas exchange

Data in mm^3/hour/mg dry weight; γ designates the CO_2/O_2 quotient

CONDITION [2]	DARK			LIGHT [1]		
	O_2	CO_2	γ	O_2	CO_2	γ
A. Medium H	— 28.4	34.2	1.20	— 13.9	17.9	1.29
(+ sucrose)	— 27.0	31.0	1.15 [3]	— 14.5	22.6	1.56
	— 29.8	35.2	1.18	— 10.0	13.7	1.37
	— 28	33	1.2	— 13	18	1.4
B, Medium S	— 13.4	12.9	0.96	23.3	— 19.7	0.85
(— sucrose)	— 14.2	12.4	0.87 [3]	24.7	— 21.5	0.87
	— 11.8	10.2	0.86	19.5	— 15.9	0.82
	— 13	12	0.9	23	— 19	0.8
C. Medium G	— 13.9	13.3	0.96	33.0	— 29.9	0.91
(— sucrose)	— 13.8	13.5	0.98	35.4	— 33.0	0.93
	— 14	13	1.0	34	— 31	0.9

[1] At saturating intensity; cf. figure 3.

[2] Cells grown in light in the medium indicated and transferred directly or with dilution in the same medium.

[3] By the indirect method; other values of respiration by the direct method.

succinic, pyruvic, malic, and glycolic acids. Only small increases (1 to 1.5-fold) followed addition of glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, hexose diphosphate, dihydroxyacetone, and lactic, citric, and phosphoglyceric acids. No substrate was found which would give greater stimulation than that of glucose.

In cells incubated in the light without carbon source other than carbon dioxide a net O_2-evolution and CO_2-uptake of

photosynthesis may be observed in light (table 4 B, C). However, the observed rates of photosynthesis are not steady but decrease slowly after the first 50 or 60 minutes. All of the photosynthesis data presented for Ochromonas describe behavior during the first hour and therefore represent maximum rates.

The low rates of photosynthesis of Ochromonas led to comparison with *Chlorella pyrenoidosa* (Emerson strain) and

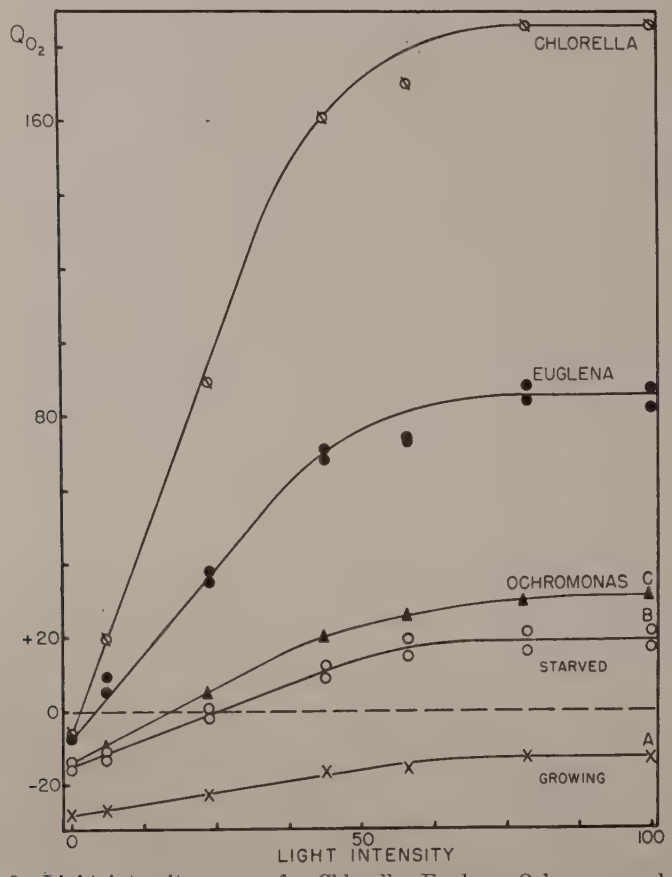

Fig. 3 Light intensity curves for Chlorella, Euglena, Ochromonas obtained in light from neon in red glass tubing ($\lambda > 640$ mμ). Intensity 100 corresponds to 16,000 ergs cm^{-2} sec.$^{-1}$ for λ 640–700 mμ. For Ochromonas cells grown and measured in medium H + sucrose (A), medium S — sucrose (B), and medium G — sucrose (C).

Euglena gracilis, var. *bacillaris.* Figure 3 presents light intensity curves as determined in red neon light varied by neutral filters strapped to the bottoms of individual vessels. The data are not of high precision since they are obtained by the abbreviated indirect method using single vessels and assuming a value of the CO_2/O_2 quotient. However, the values at zero and maximum intensity check out against the more complete measurements of table 4 and only intermediate values contain uncertainties in the proper values of the CO_2/O_2 quotient used for calculation. It will be seen that with glucose available Ochromonas never attains a compensation point. Cells lacking an available organic carbon source, and previously adapted to this condition, have a lower respiration and increased photosynthesis so that they do reach a net positive O_2-evolution. When compared with similar curves for Euglena and Chlorella it is not surprising that Ochromonas can support only marginal growth by photosynthesis alone.

4. Pigment studies. The pigments of Ochromonas extract readily and completely in cold acetone or methyl alcohol to give colorless cells and green extracts without evidence of the original brownish coloration of the cells. Comparative absorption curves for the pigments extracted by methyl alcohol and transferred to ethyl ether are presented in figure 4. The curves have been adjusted vertically by factor multiplication to coincide at the 663 mμ red peak and have no significance as to original concentrations. The only marked differences, which occur in the 630–650 mμ region are interpreted as decreasing concentrations of chlorophyll *b* in passing from Chlorella to Euglena to Ochromonas. Ochromonas shows no evidence of chlorophyll *c* with absorption peaks at 627 and 580 mμ; nor was any evidence of chlorophyll *c* found by the chromatographic procedures used by Strain and coworkers ('42, '43) to demonstrate its presence in the closely related diatoms and in a dinoflagellate.

Chlorophyll concentrations in Ochromonas and Euglena were determined from the optical densities at 663 and 645 mμ

observed by a Beckman spectrophotometer upon extracts obtained quantitatively in 80% acetone (cf. Arnon, '49). Since Chlorella is not extracted quantitatively by acetone, its pigments were extracted in hot methyl alcohol, trans-

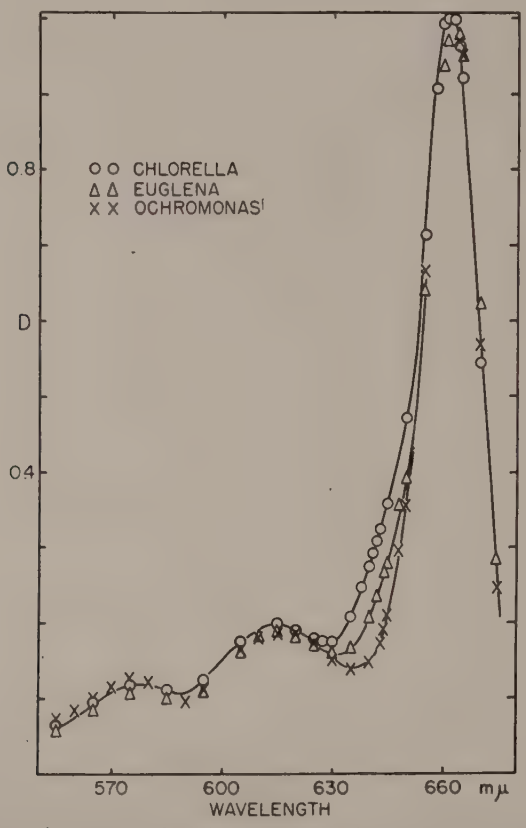

Fig. 4 Absorption curves for the pigments of Chlorella, Euglena, and Ochromonas extracted in methyl alcohol and transferred to ethyl ether. All curves adjusted vertically by a factor to give OD = 1.0 at the red peak.

ferred to ethyl ether, and concentrations determined from optical densities at 663 and 644 mμ. Cross checks of the two methods on Ochromonas and Euglena gave reasonable agreement. The data are presented in table 5. The negligibly small values for chlorophyll *b* in Ochromonas may be taken

as presumptive evidence for the lack of this component. For Chlorella, Euglena, and the three cell preparations of Ochromonas there is a direct relationship between the chlorophyll concentrations of table 5 and the initial slope and maximum rates shown by the light intensity curves of figure 3.

TABLE 5

Chlorophyll content of Chlorella, Euglena, and Ochromonas in % dry weight

	CHLOROPHYLL *a*		CHLOROPHYLL *b*		TOTAL CHLOROPHYLL		
	Ether [1]	Acetone [2]	Ether [1]	Acetone [2]	Ether	Acetone	Av.
Chlorella	3.15	...	1.00	...	4.15	...	4.2
Euglena	1.36	1.44	0.25	0.27	1.61	1.71	
	...	1.58	...	0.30	...	1.88	1.7
Ochromonas							
A. Medium H	0.13	0.17	0.006	0.01	0.14	0.18	
(light,	...	0.26	...	0.02	...	0.28	
+ sucrose)	...	0.24	...	0.02	...	0.25	0.2
B. Medium S	...	0.89	...	0.03	...	0.92	
(light	...	0.81	...	0.05	...	0.86	0.9
— sucrose)							
C. Medium G	...	1.16	...	0.08	...	1.24	1.2
(light							
— sucrose)							

[1] From OD at 663 and 644 mμ using $a_a = 95.0$ and $a_b = 5.12$ at 663 mμ and $a_a = 15.4$ and $a_b = 57.5$ at 644 mμ (Koski, '50).

[2] In 80% acetone from OD at 663 and 645 mμ using $a_a = 82.0$ and $a_b = 9.27$ at 663 mμ and $a_a = 16.75$ and $a_b = 45.6$ at 645 mμ (MacKinney, '41).

DISCUSSION

It has been demonstrated that Ochromonas possesses a photosynthesis, similar in its over-all characteristics to that of green plants, but quantitatively of such low rate that it will support only marginal growth. Several of the photosynthetic characteristics of the organisms are anomalous or, at least, not easily explained.

The low and variable chlorophyll content of Ochromonas merits further attention. When cells growing in medium H with sucrose are transferred to medium G without any or-

ganic carbon source, there occurs a 6-fold increase in chlorophyll content (cf. table 5). This may be some kind of an adaptive phenomenon. Alternately it might be explained in terms of a limiting rate of chlorophyll synthesis, independent of the condition varied, which at the 5 times shorter generation time maintained by sucrose allows synthesis of only one-sixth as much chlorophyll per dry weight.

TABLE 6

Comparison of rates of photosynthesis observed in manometric experiments with those calculated from maximum growth rates

ORGANISM	%C	CALCULATED FROM GROWTH RATE			MANOMETRIC MEASUREMENT
		k	k'[1]	Q_{CO_2}[2]	Q_{CO_2}[3]
		$\log_{10}$ day^{-1}	mg cells mg^{-1} hr.$^{-1}$	mm^3 CO_2 mg^{-1} hr.$^{-1}$	mm^3 CO_2 mg^{-1} hr.$^{-1}$
Chlorella[4]	49	0.87	0.083	77	168
Euglena[4]	48	0.43	0.041	37	80
Ochromonas	(48)[5]	0.10	0.010	9	32

[1] $k' = k \times 2.303 \times 1/24$.

[2] $Q_{CO_2} = k' \times \%C/100 \times 1870$ mm^3 CO_2/mg C.

[3] Values observed in this study, not corrected for respiration.

[4] Growth rates and %C based upon Myers and Johnston ('49) and Cramer and Myers ('52) and other unpublished data.

[5] Value assumed, pending results of cell analyses.

A problem, still more serious in Ochromonas than in other algae studied, is that the maximum rate of photosynthesis permitted under steady-state conditions of logarithmic growth is much less than that observed in short-time manometric experiments. The data of table 6 illustrate the problem. With the assumption that excretory products are negligible (cf. Myers and Johnston, '48, for Chlorella and table 3 for Ochromonas) the carbon content of the cells produced allows transformation of the specific growth rate into units of Q_{CO_2}. As observed for Chlorella and Euglena the value of Q_{CO_2} so calculated is only about one-half of that observed in manometric experiments although both values are obtained under light-saturation. The discrepancy is especially remarkable since

the manometric experiments may show a constant or even slightly increasing rate of gas exchange for several hours. An explanation applicable to Chlorella, and probably also to Euglena, is that the maximum growth rate is determined, not by rate of carbon assimilation, but by the rates of other processes necessary for cell division. Cells exposed to light saturation show a high rate of photosynthesis leading to a disproportionately high rate of starch synthesis. In time the rate of photosynthesis per unit weight or volume of cells must decrease to a value compatible with the maximum growth rate. The cause of the decrease may be attributed to glutting of the cells with storage material, or perhaps more simply, to the mere diluting out of the photosynthetic machinery by accumulating starch. Tamiya et al. ('53) have demonstrated that the cells of a population of Chlorella may be separated into categories of small "dark" cells and large "light" cells. The small cells show a maximum rate of photosynthesis about 6 times as great as that of the large cells.

In Chlorella the discrepancy between the values of Q_{CO_2} obtained from growth and manometric measurements is reasonably explained. In Ochromonas the problem is more complicated. Here the difference between the Q_{CO_2} values is greater. Here the rate of photosynthesis observed in a manometric experiment begins to fall even before the end of the first hour of saturating light intensity. And here the basic argument that carbon assimilation does not limit the rate of growth cannot be used since sucrose will raise the rate by a factor of 5. There is no simple explanation based upon experience with other algae.

Still a third problem lies in the anomaly that growing cells provided with glucose and carbon dioxide cannot evolve oxygen in the light (table 4 A) while they will grow in the light, but not in the dark, under anaerobic conditions (fig. 2). If cells growing on glucose cannot manage any net O_2-evolution, it is difficult to see how they can manage to keep themselves internally aerobic in a continually-swept atmosphere of nitrogen. It may be that Ochromonas can achieve a sig-

nificant photoreduction or photoconversion of carbohydrate to cellular material not reflected in its gas exchange.

The three anomalies of photosynthetic behavior cited above lead to the desirability of further comparative study of photosynthesis in Ochromonas.

Finally, comment may be made on the evolutionary significance of Ochromonas as a primitive organism.

> The extraordinary nutritional versatility of Ochromonas is considered to be a primitive character. From ancestors with a mixed nutritional habit, phototrophic, phagotrophic, and saprotrophic flagellates seem to have evolved, each with only one of the three modes of nutrition that are combined in Ochromonas. (Pringsheim, '52).

Ochromonas has the quintessential animal characteristics of phagotrophy and motility. Accepting the thesis, that the colorless protozoans arose from the green phytoflagellates, Ochromonas may be considered a very primitive animal which has retained only enough of its photosynthetic apparatus to sustain it between bites.

SUMMARY

1. The chrysomonad *Ochromonas malhamensis* may be cultured in a defined medium containing inorganic salts, the growth factors thiamin, biotin, and cobalamin, several amino acids, and a carbon source such as glucose or sucrose. On such media the generation time is about 14 hours. The medium may be simplified further by elimination of all amino acids with only minor decreases in growth rate. When all utilizable organic carbon is eliminated, light and carbon dioxide alone will support marginal growth with a generation time of about three days.

2. Experiments with large batch cultures of several days duration provide balances between glucose used, cells produced, and the carbon dioxide and oxygen exchange. In the presence of glucose light causes only small changes of doubtful significance. In the absence of glucose an oxygen evolu-

tion and carbon dioxide uptake is demonstrated, but only at a low rate compatible with the low growth rate observed under similar conditions.

3. In manometric experiments a net oxygen evolution and carbon dioxide uptake is obtained only in the absence of a utilizable organic carbon source, and then only at a rate very small compared to the rates exhibited by Chlorella or Euglena.

4. Pigment studies are interpreted as demonstrating absence of chlorophylls *b* and *c*. The content of chlorophyll *a* is very low in cells provided with glucose and increases by a factor of about 6 times in cells forced to grow by photosynthesis alone.

5. The data are interpreted as demonstrating that Ochromonas has a photosynthesis, similar in its over-all aspects to that of other green plants, but with several characteristics which lead to expectation of peculiarities in details.

6. In the spectrum of relationships between autotrophic and heterotrophic nutrition found among the algae Ochromonas lies close to the heterotrophic end of the scale. Present evidence confirms the thesis of Pringsheim that photosynthesis makes only marginal contribution to the versatile and probably primitive nutrition of Ochromonas.

ADDENDUM

Since preparation of this manuscript in 1954 additional studies have been made which bear upon peculiarities of photosynthesis in Ochromonas. Vishniac (personal communication) has observed a photoconversion of isopropyl alcohol to acetone. Reazin (Plant Physiol., in press) has suggested that the low rate of photosynthesis is attributable to limited synthesis of the pentose moiety required for carbon dioxide fixation.

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DISCHARGES IN MOTONEURONS OF CICADA

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FIVE FIGURES

INTRODUCTION

Considerable information on the reflex mechanism has been obtained through recording electrical potential changes in invertebrate ganglia (Bullock, '47; Prosser, '50; Roeder, '53). The neurons in these animals are mostly monopolar and thus quite different from vertebrate motoneurons. However, no attempt has been made to investigate potential changes of these monopolar neurons by intra-cellular recording. The sound producing response of the cicada seems to offer appropriate material for a study of this problem (Hagiwara, '53, in Press; Wakabayashi and Hagiwara, '53). In the present work we have attempted to throw light upon the mechanism of reflex activity including that of monopolar motoneurons and interneurons in the sound reflex of cicada with the use of an intra-cellular electrode (Ling and Gerard, '49; Nastuk and Hodgkin, '50).

MATERIALS AND METHODS

In this experiment *Graptopsaltria nigrofuscata,* one of the most common species of cicada in Japan was used throughout. After the removal of all the legs and wings, an insect was pinned dorsal side up in a shallow Petri dish which had been half filled with wax. The main sound muscle was exposed by removing the dorsal abdominal wall. The motor nerve fiber

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supplying the main sound muscle was thus found at the region where it enters the muscle near the tendinous thread which connects the muscle with the sound membrane. An abdominal branch of the nerve cord was dissected free from the inner surface of the ventral abdominal wall. The mesothoracic ganglion was exposed by removal of the dorsal cuticule, wing muscles and alimentary organs (see fig. 1). After its exposure most of the surrounding connective tissues were carefully removed with micro-shears. Insect physiological saline solution was used to cover the ganglion.

Action potentials of muscle and nerve fibers were led off by means of a pair of fine silver wire electrodes. In order to record potential changes in the ganglion, a capillary microelectrode ranging between 0.5 and 0.1 μ in tip diameter and filled with three molar KCl was used. Both the fine silver and the capillary electrodes were oriented by means of a Peterfi micromanipulator. Recording equipment consisted of a D. C. amplifier and cathode-ray-oscillograph. A cathode follower input of low grid current and reduced grid-to-earth capacity was employed for the intra-cellular recording. The electric stimulus was a square pulse of 100 micro-seconds or a condenser discharge of short time constant triggered by the sweep pulse. The experiments were carried out at a temperature of 29°–30°C. About 70 specimens were used.

RESULTS

Reflex discharge of muscle

When a single shock of sufficient intensity was applied to the central cut end of a caudal nerve branch (1 in fig. 1), a typical sound reflex was elicited. The electrical response of the main sound muscle during the reflex consisted of a repetitive spike discharge as shown in figure 1. Each muscle spike corresponded to one impulse of the single motor nerve fiber which innervates the whole sound muscle.

Change of shock intensity altered the number of spikes set up by the shock. At a low intensity the number was one. A

single shock at higher intensities sometimes elicited a series of 10 or more spikes (fig. 1). In spite of the number of spikes being increased, the greater intensity did not result in a large rise in the frequency of spike discharge; this remained fairly constant at about 100 per second.

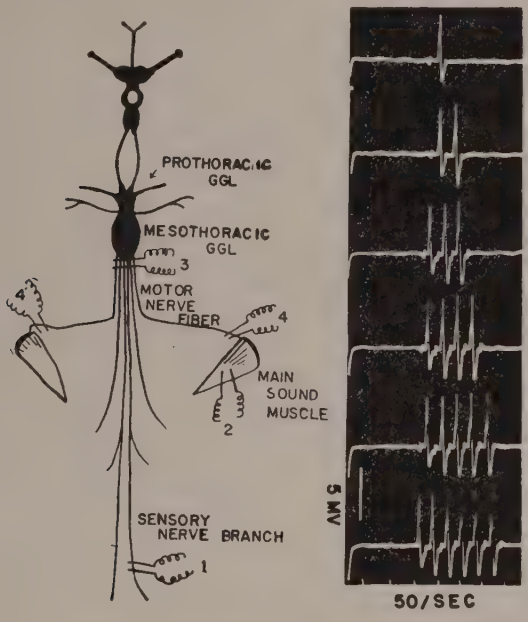

Fig. 1 Left: A schematic illustration of the nervous system of the cicada. 1, 2, 3, 4 and 4' indicate the positions of electrodes applied. Right: Reflex discharges of the main sound muscle, recorded with electrodes at position 2, set up by a single stimulation made to a caudal sensory nerve branch (position 1). The stimulus intensity increases from upper to lower. Small spikes seen among the large are those of the contra-lateral sound muscle.

The latent period of the first spike was usually very long, ranging between 50 and 60 msec. It sometimes fluctuated even at a constant stimulus intensity. By statistical treatment it was found that the variation of latency very often consisted of integral multiplications of a certain time interval which was about 10 msec or equal to the average interval of motor discharges. This suggests that such a variation may be due to occasional failures of transmission of early motor impulses.

Similar failure of transmission was also expected from the fact that, during the reflex discharge of the main sound muscle, the dropping out of a spike was often observed. These failures do not seem to take place at the neuromuscular junction, because a similar spike failure was also observed in the motor nerve fiber supplying the main sound muscle. As described in a later section this seems to occur at a more proximal part of the neuronal chain of this reflex.

Each insect has a pair of main sound muscles. When discharges of these two muscles were recorded simultaneously, every spike of one muscle was obtained in midinterval of the contralateral muscle. In figure 1 the small spikes seen among the large are those of the contralateral muscle. As described in the previous paper (Hagiwara, in press) this phenomenon is not due to the difference between the peripheral nervous conduction time for the muscles. Nerve impulses for them leave the mesothoracic ganglion already alternately. This was confirmed by simultaneous recording of impulses in their motor nerves at the place where they leave the ganglion (Hagiwara, in press).

Central reflex time

In order to obtain the central reflex time, it is necessary to estimate the time spent in peripheral conduction. A brief stimulus applied to the efferent nerve fiber at the region where it leaves the mesothoracic ganglion (3 in fig. 1) caused an action potential of the main sound muscle with a latency of about 9 msec. When the impulse of the nerve fiber was recorded at its entry to the muscle (4 in fig. 1), the latency was 3 msec. This indicates that the conduction time from the ganglion to the above nerve entry is about 3 msec, while 6 msec is spent for the impulse to reach the muscle from the nerve entry and to set up the muscle action potential. Similar results were obtained with the efferent nerve fiber stimulated at its entry to the muscle and recorded near the ganglion.

When the afferent fibers were stimulated close to the ganglion, a latent period of about 40 msec was obtained for the

first reflex spike of the muscle instead of its ordinary value of about 60 msec. From this result we estimated that the afferent conduction time was 10–20 msec. This value corresponded to a conduction rate of about 0.5–1 m per second. Such a slow rate may be due to the small diameter of these afferent fibers which probably innervate hair sensillae on the caudal part of the body surface. By eliminating the peripheral conduction time we obtained a value of 30–40 msec for the central reflex time.

Response of motoneuron

In order to examine potential changes in single neurons of the ganglion, the intra-cellular electrode was employed. In the course of slowly pushing the electrode into the ganglion, resting potentials, some of which were accompanied by positive spikes were observed from time to time as the electrode tip penetrated one cell after another. In the following experiments the spikes which appeared in response to a stimulus just strong enough to elicit the discharge of the main sound muscle were considered to be related to the sound reflex.

When potential changes in the ganglion were examined during antidromic stimulation of the efferent nerve fiber supplying the main sound muscle we could not find any response to the stimulus until the tip of the electrode reached the caudal end of the ganglion. The first observable response was a negative spike of a few mV which was not associated with the resting potential (upper three records in fig. 2B). The latency of this response was always about 3 msec, which corresponded with the calculated efferent conduction time. With further careful movement of the electrode, we often obtained a sudden resting potential followed by a large positive response to stimulation. Figure 3B shows such a transition from external to internal recording. The resting potential of this neuron was not larger than 60 mV, and an antidromically elicited spike was observed rising to a peak of about 75 mV in 1 msec and falling in another 2 msec. The intra-

cellular potential as well as the externally recorded spike behaved strictly in an all-or-none manner when the stimulus intensity was varied. This shows that the neuron concerned is the motoneuron of the main sound muscle on one side. As each of the main sound muscles is innervated by a single ef-

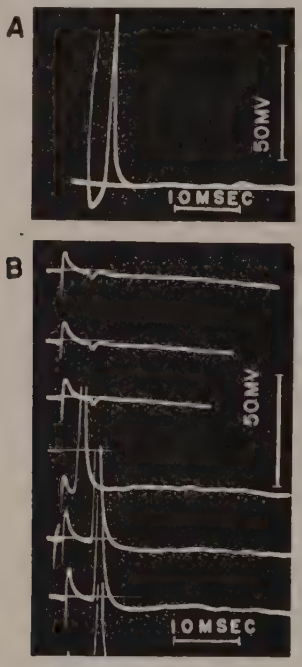

Fig. 2 Action potential of the motoneuron of the main sound muscle set up by antidromic stimulation.

A Intracellular recording. The peak voltage measures 73 mV.

B Transition from external to internal recording. The upper three curves show small, negative, externally recorded potentials and the lower three large, positive, intracellular potentials. Resting potential at the instant of penetration of the electrode is about 60 mV.

ferent nerve fiber, there should be only one motoneuron corresponding to each of the muscles. In spite of the number of neurons being very small the penetration of the electrode tip into the neuron could be accomplished without any serious difficulty. This may be due partly to the large amplitude of the externally recorded action potential and also partly to a large

diameter of the neuron soma. In fact histological preparations showed groups of ganglion cells whose diameters ranged between 60 and 70 μ, and two of these are probably motoneurons of the main sound muscles.

When stimulus was changed from antidromic to orthodromic, a reflex response of the motoneuron was observable (fig. 3). The reflex response consisted of several spikes, just like that of the main sound muscle. There were no obvious

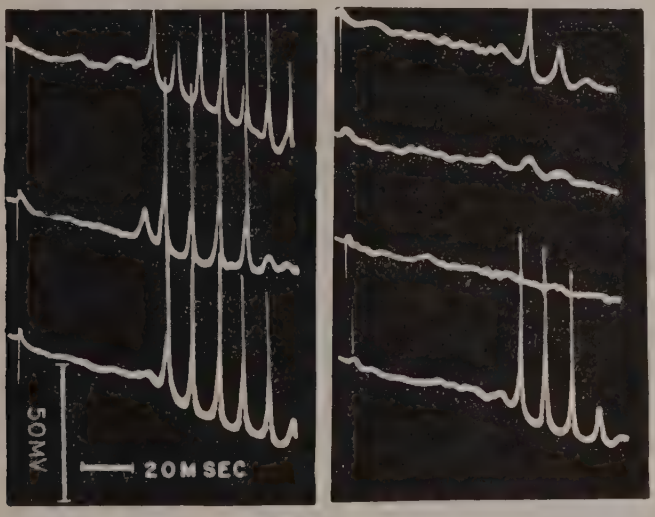

Fig. 3 Reflex discharges of a motoneuron of the main sound muscle. The postsynaptic potential always precedes each spike There are also seen abortive synaptic potentials among these spikes.

differences between the shape and height of the spikes generated by the synaptic excitation and those evoked by antidromic invasion except that the former were invariably preceded by a step which indicated a transition from the local to the propagated potential. The local potential generally flared up into a spike when it reached a critical level. But sometimes a few of them failed to grow up into a full spike. Such abortive potentials very often appeared at an early phase of the reflex response and were also found at its later phases. The existence of an abortive potential leads one to suppose

that some of the impulses initiated at certain premotor neurons failed to excite the motoneuron. From this supposition it is conceivable that the site of the failure of transmission previously mentioned is the premotor motor synapse.

The insertion of the electrode sometimes caused a rhythmic firing of the motoneuron. In such instances the intra-cellular potentials consisted of a series of local potentials some of which flared up into a propagated potential.

Responses of internuncial neurons

Antidromic stimulation evoked only the motoneuron discharge. But stimuli applied to a caudal nerve branch or cord caused other kinds of responses which seemed to be related to the sound reflex. These were classified by their patterns into several types and two of the most commonly encountered types will be described next.

The first was characterized by a short latency as well as by a strict one to one relation between action potential and stimulus. Figure 4C shows one of the responses of this type. The latency of about 20 msec is not so much greater than the peripheral afferent conduction time. This suggests that the neuron with this type of response may be situated at the most peripheral part of the neuronal chain of the present reflex.

The other common pattern consisted of a group of spikes which increased in number as the intensity of the orthodromic stimulus was raised. This response had a latency of about 30 msec. The pattern of this response is very similar to that of the motor response, but the impulse frequency of the motor response was always about 100 per second, while in the present case it was about 200 per second (fig. 4A and B). The fact that each potential has a small amplitude and occasionally a negative phase, indicates that it is an externally recorded action potential. But even under such a condition the first spike behaved in all-or-none fashion to the change of stimulus intensity. Sometimes the tip of the electrode en-

tered such neuron, and in this case, a resting potential of about 25 mV and an action potential without any obvious overshoot were observed (fig. 4D). This may be due not to the small amplitude of the true action potential but to the

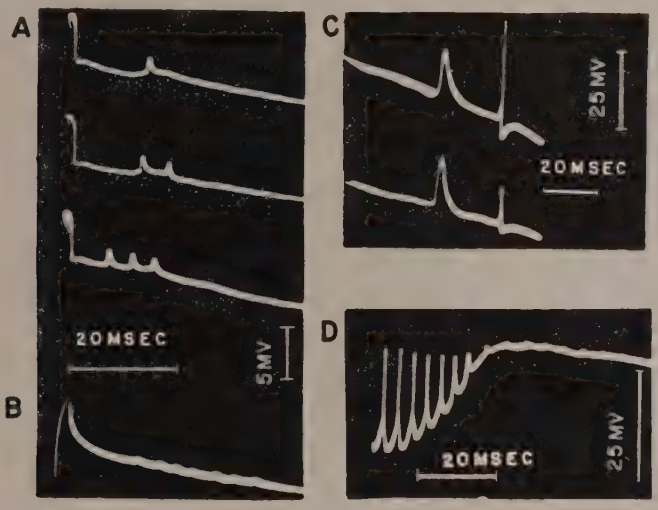

Fig. 4 Internuncial responses set up by electrical stimulation of the afferent nerve (A,B,C) or by touch stimulation to the body surface of the cicada (D). In A and B the stimulation has been made near the ganglion, which procedure reduces the latency of the first spike by about 20 msec.

A The stimulus intensity increases from upper to lower.

B A single stimulus elicits a small repetitive discharge, the number of which amounts to seven in this record.

C Single spikes preceded by the stimulus artifact. In this case the resting and action potentials were quickly reduced after the insertion of the electrode into the cell body, presumably because of its injury.

D Decrease of the resting and action potentials seems to be due to escape of the electrode from the cell body due to movement of animal.

possibility that the insertion of the electrode tip causes damage to the nervous element so that the full potential can not be recorded by this method.

Origin of motor discharge of 100 per second

Intra-cellular recording from motoneurons during the reflex discharge raises the question whether the motor rhythm

is originated at the motoneuron itself. The next thing to be examined is whether or not the spike of the motoneuron can antidromically conduct to the place where the rhythm is originated. For this purpose the effect of an antidromic impulse upon the reflex discharge of the motoneuron was ex-

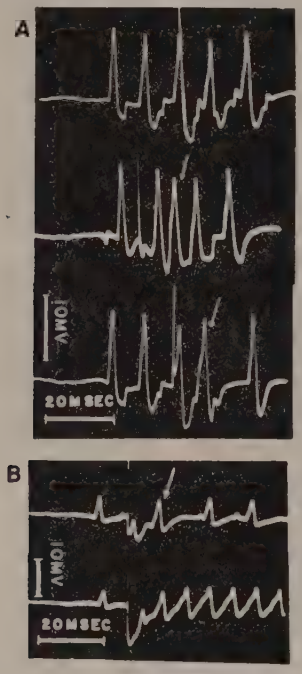

Fig. 5 A Effects of antidromic stimulation upon the reflex discharge of the sound muscle.

Upper: The case of absence of effect due presumably to the stimulation occurring in the refractory period.

Middle: An extra-spike (indicated by an arrow) that appears between two successive reflex spikes.

Lower: An extra-spike (indicated by an arrow) followed by the so-called compensatory pause.

B Effects of the direct stimulation of the mesothoracic ganglion upon the reflex discharge of the sound muscle.

Upper: The stimulus is applied antidromically during the reflex discharge as in A and the efferent discharge shows an extra-spike but there is no change in its frequency.

Lower: The stimulus is applied directly to the mesothoracic ganglion. The frequency of discharge becomes twice that before stimulation.

amined by recording the action potential of the main sound muscle. The motor nerve fiber was stimulated near its exit from the mesothoracic ganglion. Such a stimulus alone always caused an action potential of the motoneuron within half a millisecond. When the stimulus was applied during the refractory period of the motoneuron caused by a preceding reflex spike, no noticeable change occurred in the motor discharge (upper in fig. 5A), while the stimulus during the remaining phase set up an extra-spike of the neuron (middle and lower in fig. 5A). The extra spike did not change the phase of the following pattern of the reflex discharge (as shown in fig. 5A). This phenomenon is quite similar to that observed with an extra-systole of the ventricle of mammalian heart. The so-called compensatory pause was also obtained in some cases of the present experiment. A short time interval between the extra spike and the next expected impulse seems to result in this pause. These results indicate that a spike of the motoneuron can not propagate into the origin, therefore, the motor rhythm must be originated at a premotor neuron.

DISCUSSION

The resting potential of the insect motoneurons examined in this investigation was about 60 mV, while the peak voltage of its action potential was about 75 mV. According to Brock, Coombs and Eccles ('52) the resting and the action potential in certain vertebrate motoneurons are 80 mV and 120 mV respectively. They have also obtained small values from direct recording but arrived at an estimate by adding a certain amount to compensate for a possible reduction of potential due to injury. Therefore, the observed value may not be so much different in the two cases. Furthermore it is not necessarily to be expected that the ratio of internal to external potassium should be constant for all nerve cells. This ratio may determine the resting potential (Hodgkin, '51). Many insects are known to have low ratios; this cicada has not been examined in this respect. We have sometimes recorded rest-

ing and action potentials less than those mentioned above. This occurred less often in motoneurons than in other types and was probably due to injury of the neuron in the process of inserting the electrode. This effect may be very serious in small nerve cells such as internuncial neurons.

Brock, Coombs and Eccles ('52) found a brief step or double inflection on the rising phase of the antidromic spike of a vertebrate motoneuron. They have interpreted this as a transition of the excitatory process from the non-myelinated region of the motor axon to the neuron soma. Such a step has not been observed in the present material and may be related to the fact that the motoneuron of insects has little or no axon hillock (Hanström, '28).

The motor rhythm of the reflex discharge, we conclude, is originated at the premotor neuron. A similar pattern was also found in the reflex discharge of the motor center of the electric organ in a certain fish (Fessard and Szabo, '54).

One of the most striking properties of the present reflex dicharge is the alternate innervation of the two main sound muscles. To account for this the following two mechanisms may be considered. (a) Each impulse of one motor system may be supposed to start with a certain delay after the initiation of each impulse of the other system and the delay happens to be about 5 msec, i.e. half of an ordinary impulse interval. (b) The two motor systems are equally controlled by a single pacemaker. This alternative would require either that one motor center (motoneuron and immediate premotor neuron or neurons) had a 5 msec longer delay than the other or that the pacemaker fires at twice the frequency of the motoneurons and only every other time controls a given motor center. Furthermore it would be necessary to postulate some mutual inhibitory interaction of the motor centers in order to insure their responding alternately. This mechanism (B) seems most likely. A 200 per second rhythm has in fact been found in cells regarded as internuncial in the sound reflex path. It has been possible occasionally to drive a motoneuron at 200 per second by applying a brief stimulus to the mesothoracic

ganglion during the reflex discharge as shown in figure 5B. Mechanism A would require two premotor neurons or centers and neither of these could ever fail or both motoneurons would fail to fire. We observe that during a reflex discharge one motoneuron may miss several cycles without any sign of failure in the other one. When the first one resumes firing it is always in mid-interval of the second, bespeaking an active depression of its likelihood of firing in phase.

SUMMARY

1. Investigation was made of the neural mechanism of reflex activity of the sound reflex of cicada, the effector of the reflex being the main sound muscle which is innervated by a single motor nerve fiber, its receptors being hair sensillae on the body surface, and its center in the mesothoracic ganglion.

2. A single stimulus applied to the nerve from the hair sensillae caused a grouped discharge of the sound muscle. The number of spikes in the group increased with increase of the stimulus intensity, while the frequency of spikes was almost unchanged by it, being at about 100/sec.

3. The two sound muscles, left and right, were found to be alternately excited.

4. 30–40 msec was obtained as a value of the central reflex time.

5. The resting and action poential of motoneurons of the sound muscle were 60 and 75 mV respectively (maximum observed values; intracellular electrode). Reflex discharges of the motoneuron were repetitive spikes of about 100/sec, each of which was preceded by a small local potential. This potential often failed to set up a spike.

6. Two distinct types of response probably in internuncial neurons were observed, one showing a single spike to a single afferent stimulus and the other repetitive spikes of 200/sec.

7. The motor rhythm of about 100/sec was concluded to be originated at the premotor neuron. And the alternate excitation of the two sound muscles was considered to be controlled by a common pacemaker and some mutual inhibition.

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ON THE PRESENCE OF PNA IN CERTAIN ION-AGGREGATED PARTICLES FROM ARBACIA EGG HOMOGENATES [1]

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TWO FIGURES

INTRODUCTION

In the presence of ionic calcium in very low concentration, the cytoplasm of the sea urchin egg undergoes changes which result in a sharp increase in viscosity. In the normal cell, such an increase in viscosity follows fertilization and precedes cytokinesis. The "mitotic gelation" is a specific case of this very general response of the cell to stimulation. The cytoplasmic sol-gel transformation has provided the basis of one influential theory of cell stimulation and division, the so-called "colloid chemical" theory (Heilbrunn, '52). Significantly, phenomena supposed to depend upon changes in the colloidal properties of cytoplasm are supressed in the absence of Ca. Thus, cells which are injured, e.g., by crushing, suffer an extreme form of colloidal rearrangement which has been called "protoplasmic clotting." The reaction proper has been named the Surface Precipitation Reaction (abbreviated SPR). There are many reasons for considering this coagulation to be an extreme form of the normal cytoplasmic sol-gel transformation (Heilbrunn, '28).

The response to recalcification of sea urchin egg homogenates, prepared with initial removal of Ca, involves many

[1] Preliminary notes dealing in part with this research have appeared in Biol. Bull., *107:* 298 (1954) and in Anat. Rec., *120:* 801 (1954).

reactions which mimic those accompanying the normal sol-gel transformation and the SPR (Gross, '54a; Hultin, '50a, '50b). Among the reactions is a rearrangement of the proteins, leading to an increased sedimentability of one or more fractions. This increased sedimentability is a manifestation of an aggregation or polymerization process. These changes may represent a molecular basis for the events of the SPR and, perhaps, for the normal cytoplasmic "gelation."

Preliminary spectrophotometric evidence bearing upon the nucleoprotein nature of the aggregating material has been presented elsewhere (Gross, '54a). This report presents further data concerning the characterization of the aggregating material and the mechanism of the reaction which underlies the process.

METHODS

Homogenates of eggs from the sea urchin, *Arbacia punctulata,* were prepared in the manner described previously in some detail (Gross, '54a). This preparation is free of ionic Ca, and probably of the other divalent cations normally present. Although the homogenization medium is ionic (0.5 M KCl or buffered 0.5 M KCl), there is no excessive aggregation of the particulates, and indeed, even the heavy pigment granules settle out only after many hours. Earlier studies (Harris, '43) have shown that homogenization procedures of this type result in the lysis of a relatively small percentage of the pigment granules.

In most of the experiments, a partial fractionation was performed prior to the actual experimental manipulation. The homogenates were centrifuged, after chilling to 2°C., at 1500 $\times$ g for ten minutes. This step removes all of the red, echinochrome-containing pigment granules together with most of the yolk. In general, the homogenates were prepared from one volume of centrifugally packed, jellyless eggs and four volumes of KCl medium. This material, after separation of the pigment and yolk, was turbid and faintly yellow-orange. Its pH was 6.5–7.0.

The partially fractionated homogenates were treated with various small quantities of calcium chloride as described below. In every case, simultaneous controls were run in which the increment of ionic strength contributed by the added Ca in the experimentals was supplied by suitable amounts of KCl in the controls. Thus, none of the effects to be described is attributable to the general charge properties of the ionic atmosphere alone, and the *specific* properties of the divalent ion are selected in virtue of the experimental design.

After incubation of these systems for suitable periods under the chosen conditions, they were rapidly diluted with ice-cold distilled water, usually to about ten times the volume of the incubation system. Extraction of soluble materials was accomplished by storage at 1°C. for periods of from a few minutes (experiment 54III, *vide infra*) to several hours.

The thoroughly chilled material was then placed in plastic centrifuge tubes and spun at 25,000 × g in a Servall type SS-1 angle-head centrifuge for from 5 to 20 minutes. Analytical work was performed on the supernatant solutions thus obtained, and in one case, on the residues as well. Total protein was determined by a quantitative Biuret technique (Fine, '35) and the results were independently checked in several cases by micro-Kjeldahl nitrogen estimations. The protein and nucleic acid moieties of the water extract were separated by means of an adaptation of the technique devised by Schneider ('45), and following this author, PNA was determined by the orcinol reaction of Mejbaum and DNA was assayed with the diphenylamine reagent of Dische. In the whole homogenate, however, and often in the pigment-less fraction used in the present experiments, there are found interfering materials which make the diphenylamine reagent quite unreliable. DNA was ultimately, as a result of this condition, determined by difference. PNA was, as usual, determined by the orcinol reaction, and then total nucleic acid was estimated by calculation from nucleic acid phosphorus values (P estimated by the method of Fiske and Subbarow,

'25). DNA was determined as the difference between PNA and total nucleic acid.

Since all methods agree in showing virtually no DNA in the system free of nuclei and large particles, the relative imperfection of this method was not considered of sufficient moment to warrant employment of the more elaborate methods currently in use, and ultimately the large changes in total nucleic acid observed spectrophotometrically (Gross, '54a) were considered to represent changes in PNA.

Ca was determined by means of a slight modification of the method of Fales ('53), which involves a spectrophotometric titration of the calcium with EDTA (ethylenediamene tetraacetic acid) in the presence of murexide (ammonium purpurate) as indicator. This reaction was standardized for known amounts of calcium, both alone and in the presence of various quantities of added protein (gelatin and egg albumin). Results were highly reproducible, independently of the presence of (Ca-free) protein. The technique used modifies that of Fales only in the adjustment of reagent concentrations (the original method is designed for the determination of serum Ca) and in the substitution of a colorimeter reading in arbitrary density units for a transmission-read spectrophotometer. As in the original report of Fales, the endpoints were determined graphically by a slope-intercept method.

Electrophoresis of the centrifuged and dialyzed water extracts described above was carried out in the Perkin-Elmer portable instrument in triethanolamine buffer at pH 7.6 and ionic strength 0.1. Conductivities were determined with a simple bridge circuit containing a micro cell as one arm.

RESULTS

In a previous publication (Gross, '54a) it was suggested that the material which precipitates from the sea urchin egg homogenate under the influence of calcium is rich in nucleic acid. This was assumed on the basis of differences in the shapes of absorption spectra of the water extracts of experi-

mentals and controls. This suggestion is strengthened by the results of certain rather different experiments of Lindvall and Carsjö ('51) to be discussed below.

Table 1 shows the result of three separate experiments in which the PNA remaining soluble in the supernatants was determined after addition of Ca to experimentals and of K of equal ionic strength to controls. The incubation times varied from ten minutes to twelve hours. The temperatures of incubation and the final concentrations of added Ca in the experimentals are shown. Experiment 54I was performed upon a whole homogenate, while experiments 54IV and 54V

TABLE 1

EXPERIMENT	TEMP. °C.	INCUB. TIME	CONC. Ca mM/l	PNA CONTROL mg	PNA EXP. mg	% CHANGE
54I (Whole homogenate)	18	10 min.	44	0.180	0.103	—42.8
54IV (Supernatant I)	18	120 min.	53	0.710	0.513	—27.8
54V (Supernatant I)	1	12 hrs.	49	0.219	0.132	—39.7

were done with "supernatant I," which is the homogenate freed by centrifugation of its pigment granules and of most of its yolk granules, as described under METHODS. In the whole homogenates, there is present a substance which sometimes interferes with the production of the usual green color in the orcinol reaction. This material, which is not glucose, seems to reside in the pigment granule fraction, and it converts the orcinol color to a rusty brown. The absorption spectra of this "blocked" color and of the normal color were recorded. Both have a sharp maximum at about 670 mμ, but the normal color is represented by a trough at 550, whereas the "blocked" color in an extract of the whole homogenate has a second local maximum at that point.

It was concluded from runs with samples of known quantities of ribose with and without the contaminating material that the absorption due to the ribose derivative at 670 mμ is unaffected by the presence of the subsidiary component (and its absorption band), and that, therefore, the PNA determinations upon whole-homogenate extracts were admissible.

The table shows that in the whole homogenate 44 mM/l of calcium acting at 18°C. for ten minutes produced a loss of 42.8% in the water-extractable PNA. Calcification with 53 mM/l in experiment 54IV resulted a loss in solubility of 27.8% from water extracts of supernatant I after two hours

TABLE 2

EXPERIMENT	CONC. Ca mM/l	TEMP. °C.	INCUB. TIME min.	PROTEIN mg Control	PROTEIN mg Exp'tl.
54III	9.8	23.5	60	49.6	40.6
54VI	28.2	18.0	10	12.6	8.25

EXPERIMENT	PNA mg Control	PNA mg Exp'tl.	% CHANGE IN PROTEIN	% CHANGE IN PNA
54III	1.60	1.19	— 18.2	— 25.6
54VI	0.666	0.337	— 34.5	— 49.5

at 18°C. In experiment 54V there was a loss of 39.7% in water-extractable PNA after incubation with 49 mM/l of Ca for twelve hours in the refrigerator at 1°C.

In all of the present experiments, the pellets resulting from the centrifugation at 25,000 × g differed characteristically between the experimentals and the controls. The control sediments were gelatinous and brown. Those obtained in the Ca-treated experimentals also showed a brown, gelatinous layer, but had always, in addition, an overlayer of dark cherry-red material. This probably represents an aggregate of the fraction which becomes sedimentable in the presence of Ca, and the distinctly new color suggests that the extra sediment is more than simply an additional quantity of the same fraction that sediments in the control.

Table 2 gives the results of two experiments in which the loss in solubility of protein in supernatant I was determined along with the loss in PNA. In experiment 54III, supernatant I was treated with 9.8 mM of Ca per liter at 23.5°C. for one hour. Extraction of the material with water was followed by analysis of protein and PNA in the extract, with the following results: Protein, 18.2% less extractable in experimental than in control, and PNA, 25.6% less extractable in experimental than in control.

In experiment 54VI, which was run with a higher concentration of calcium (28.2 mM/l) at a somewhat lower temperature (18°C.) and for a shorter interval (10 minutes), the

TABLE 3

Experiment 54III

SAMPLE	TIME min.	PROTEIN mg/ml	PNA mg/ml	Ca ADDED μg/ml	Ca RECOVERED (SUPERNATANT) μg/ml
A	0	49.6	1.60	0	0
B	15	45.4	1.41	51.3	53.8
C	30	45.1	1.27	51.3	52.9
D	60	40.6	1.19	51.3	53.8

solubility losses in the experimental were respectively 34.5% and 49.5%. Evidently the PNA is being lost from the extract faster than is the protein, in both experiments.

Experiment 54III, whose results are completely tabulated in table 3, had two objectives. One goal was simply to determine how rapidly the precipitation or aggregation reaction occurs, i.e., to explore the possibility of later more elaborate kinetic study of the reaction(s). The second was to investigate the extent of binding, if any, of the initiator ion, Ca^{++} by the material which aggregates under its influence. The presence of all or most of the added Ca in the precipitates would indicate as a mechanism for the reaction a kind of coprecipitation between the Ca and the "nucleoprotein," probably by simple neutralization or reversal of the negative charge on the macromolecule. On the other hand, the presence

of all or most of the added Ca in the supernatant would suggest an indirect role for the ion, perhaps that of an enzyme activator (compare Gross, '54b).

The experiment was performed in the following way: One milliliter of a fresh supernatant I preparation was pipetted into each of four plastic test tubes. Calcium was added to three of the tubes within as short an interval (30 sec.) as was possible. The final concentration was in each case 9.8 mM/l. To a control tube was added sufficient KCl to bring ionic strength and volume up to the levels in the experimentals. At the end of fifteen minutes, one of the experimentals was removed from the water bath (regulated at 23.5°C.) and to it were added rapidly 10 ml of ice-cold distilled water. The tube contents were mixed by inversion. This tube and a balance were finally placed in the high-speed centrifuge and spun at 25,000 $\times$ g for five minutes. The supernatant was decanted, and both it and the sediment were saved. At thirty and sixty minute intervals, respectively, after the addition of Ca to the experimentals, a tube was removed from the water bath, its contents diluted and extracted, a balance prepared, and the two centrifuged for five minutes after reaching the relative centrifugal force of 25,000 $\times$ g. The control was processed along with the 60-minute experimental.

The supernatant fractions were analyzed in each of the four samples (control and three experimentals) for total protein and PNA. The experimental supernatants were assayed for their calcium content, as were the sediments after resuspension and solution in strong alkali.

Figure 1, prepared from the data given in table 3, shows the progress of the reaction which results in a solubility loss in both protein and PNA from the water extracts of the homogenates. The graph is a plot of percent solubility loss (i.e., change in the amount extractable), for both protein and PNA, as a function of time. The amounts of protein and PNA in the extract of the untreated control were taken as the baseline values. Plainly, the PNA becomes sedimentable faster than the protein, so that at the end of one hour of incubation,

25.6% of the initially soluble PNA has become sedimentable at 25,000 × g, whereas only 18.1% of the water-extractable protein has been similarly affected.

These data are not adequate for accurate curve-fitting and study of kinetic order, and the curves are therefore empirical and drawn with dashed lines.

The final concentration of calcium in the incubation mixtures "B," "C," and "D") was 0.00981 M (determined by a control titration with EDTA). If no Ca had precipitated in these tubes, then the expected value would be, for each

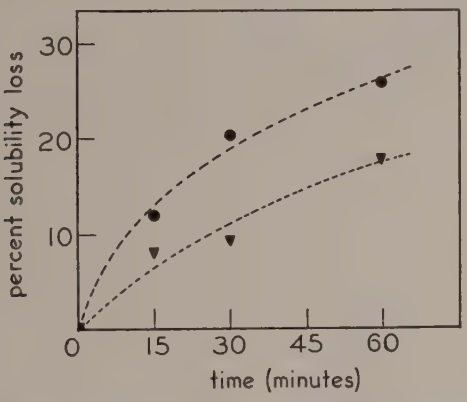

Fig. 1 Time course of aggregation, as manifested by increased sedimentability, of protein and PNA in water extracts of Ca-treated homogenate. See text and table 3. Circles: PNA. Triangles: Protein.

experimental extract, 51.3 μg/ml. This is shown in table 3 in the column headed "Ca added." The column headed "Ca recovered in supernatant" records the result of duplicate titrations with EDTA of samples of each of the experimental supernatants. Clearly, all of the added Ca remains in the soluble fraction of the system. The slight *excess* of Ca recovered (i.e., 53.8 and 52.9 μg compared with 51.3) is not explainable in a satisfactory way at present. Possibly the consistently higher value in the supernatants represents approximately 2–3 μg of Ca per milliliter of the extract which was in bound form in the original homogenate and was not re-

moved by the citrate washings of the eggs. This would suggest the presence of about 7×10^{-5} M/l of Ca remaining in the original homogenate preparation, a not unlikely quantity.

The pellets resulting from the centrifugations of each of the four samples were dissolved in strong alkali and made up to volume. Then each was separated into aliquots in which Ca was estimated. There was no Ca titratable in any case. Thus within the limits of sensitivity of the present technique, no Ca precipitated with the sediment, and all of the added ion was accountable for in the supernatant as dissolved.

The macromolecular composition of the supernatants of the extracts is of considerable interest for the present study. The following experiment was performed with the purpose of assaying electrophoretically the results of incubation of the material with Ca. Six milliliter aliquots of a fresh homogenate were incubated at 18°C. with a total concentration in Ca of 44 mM/l. The incubation interval was 10 minutes. At the end of this time, control and experimental were quickly chilled to 0°C. and placed in the centrifuge. The material was spun at 25,000 $\times$ g for twenty minutes. Aliquots of the supernatant material were removed for chemical analyses, and the remainder was, in each case, mixed with two volumes of triethanolamine buffer at pH 7.6 and ionic strength 0.1. The mixtures were then dialyzed, with stirring, at 1°C. for 48 hours, against several changes of the buffer. At the end of this period of dialysis, the material was recovered and centrifuged to remove residual turbidity. The conductivity was determined. Control and experimental were then run, within a single afternoon, in the Perkin-Elmer electrophoresis instrument. Photographs of the ascending limb of the 2 ml cell were made at intervals. The (approximate) mobilities of the several boundaries were calculated from measurements made on the negatives. The results of this experiment are given in table 4 and in figure 2.

The analytic data show the result of incubation with Ca as a loss in solubility of 31.2% of the protein and 77.4% of

the PNA. These differences are reflected with good agreement in measurements of the relative areas under the schlieren patterns. The reader should note that the total weight loss in extractable PNA is less than 2% of that in total extractable protein.

Figure 2 is made from an enlarged tracing of the pattern, representing the positions of the boundaries after 3000 seconds of migration in each case. The upper pattern is that of

TABLE 4

Experiment 54II

	EXPERIMENTAL	CONTROL	% CHANGE
Total protein (mg/ml)	6.50	9.45	—31.2
PNA (μg/ml)	21.2	93.8	—77.4
Total area under electrophoretic pattern (cm^2)	7.18	10.9	—34.1
Electrophoretic mobility — ($cm^2/v/sec \times 10^5$) of component no.:			
1		22.1	
2	13.9	12.2	
3	9.28	7.58	
4	3.35	2.82	

the control and the lower one was obtained during the experimental run. The control pattern shows good separation of four boundaries, with, possibly, a fifth close to the starting point (at the crossed lines). The approximate mobilities of these components are given in the table. It will be noted that the mobility of the small, fastest boundary is very high, and that the mobility of the main peak is also high at a pH of 7.6. A comparison of the experimental pattern with the control reveals several differences. First, the fastest component

has disappeared. Secondly, the area under the main peak has sharply diminished. Third, the other boundaries seem not to have been affected by the treatment. The calculated mobilities of the experimental peaks are all higher than those of the control, with the increment scattered about 15%. There is, at

Fig. 2 Electrophoresis of supernatants from homogenate. Upper pattern, control; lower pattern, experimental. Ascending limb after 3000 seconds. Buffer: Triethanolamine, pH 7.6, ionic strength 0.1. Arrow indicates direction of migration (anodic).

present, no complete explanation for this observation. The reduced viscosity of the experimental may, perhaps, have played a part in increasing the mobilities, but the exact contribution of this parameter cannot be estimated.

There is no doubt, however, about which peaks correspond in the two experiments, nor about which have undergone changes in the experimental system. The experiment shows

that except for the disappearance of a small, very fast component from the experimental (a quantitatively insignificant event) the change has been confirmed to the large, fast peak called number 2 in the table.

DISCUSSION

In 1946, Schneider reported that Ca, at concentration levels approaching physiologic (0.033%), effected a precipitation of nucleoprotein from rat liver homogenates. Furthermore, the precipitation reaction was shown to involve particles other than nuclei and mitochondria. Schneider could precipitate 85% of the pentose nucleoproteins of a liver extract which contained only particulate material which would otherwise require very high speed centrifugation for its sedimentation. Increased sedimentability of cytoplasmic nucleoprotein particles under the influence of Ca was shown here in a material other than marine eggs. The report suggests that the reaction is immediate, and that its mechanism is probably a simple agglutination of particulate material by $CaCl_2$. This conclusion cannot be final, however, until experiments such as those herein reported are performed upon the liver material. Such experiments are in progress in this laboratory.

Precipitation of pentose nucleic acid by low concentrations of Ca has been demonstrated by Lindvall and Carsjö ('51) in extracts of frozen and dried sea urchin eggs (*Strongylocentrotus droebachiensis* and *Echinus esculentus*). They explain, on the basis of this reaction between Ca and a nucleoprotein, the solubility loss in cytoplasmic proteins thought by Mirsky ('36) to occur after fertilization. The Swedish workers believe that the Mirsky observations reflect not so much conditions associated with fertilization as they do an artefact which appears as a result of the lyophilization of eggs with their fertilization membranes intact. They propose that a reaction between occluded Ca and nucleoprotein is the underlying event, and that this is prevented if the occlusion of Ca by the fertilization membrane is prevented.

Recently, Tsuboi et al. ('54) have studied the distribution of PNA within centrifugally separated fractions of the *Arbacia* egg. These authors find the bulk of the PNA in a fraction which consists of particles estimated to have a diameter of about 3×10^{-6} cm. This estimate, based on sedimentation velocity, agrees very well with the electron microscope observations of Gross ('54a, '56), which show that the particle which aggregates and becomes sedimentable in the presence of Ca has a diameter of $3.8–4.0 \times 10^{-6}$ cm. Tsuboi et al. have also demonstrated what they interpret as a dissociation of nucleic acid from sedimentable protein particles under the agency of EDTA and of high ionic strengths of uni-univalent salts. These workers believe that Ca can participiate in or initiate a reversible aggregation of nucleoprotein macromolecular structures in the cytoplasm.

These results agree in the observation of increased sedimentability or precipitation of PNA of cytoplasmic origin in the presence of Ca. The earlier results of my own research (Gross, '54), together with the present data, reinforce this observation. There is in addition a uniform belief expressed in papers in this field that the nucleic acid is normally present in a soluble fraction made up of macromolecules or of very small "cytoplasmic particulates."

Hultin ('50a, '50b) has presented results which demonstrate the chemical similarities between events following fertilization in the sea urchin egg and those following calcification of homogenates of the egg. Gross has suggested ('54) that the homogenate experiments duplicate the principal conditions of the SPR, and that the reactions taking place in the recalcified homogenate are, in fact, identical with those which cause the SPR. Thus it has been shown that while in the SPR some fraction(s) of the cytoplasm polymerizes, or gelates, so that the broken cell may locally reconstitute itself, in the homogenate some protein fraction(s) becomes aggregated, and this is manifested by an increased viscosity of the homogenate (Hultin, '50a).

The present results prove that the aggregating material contains nucleic acid, and must, in fact, contain a considerable part of all of the soluble cytoplasmic PNA. The electrophoretic experiment lends support to this conclusion. The fraction which undergoes the solubility loss is that represented by boundary no. 2, and this has, even at a pH close to neutrality, the very high mobility of -13×10^{-5} cm^2/v/sec. Such a mobility is characteristic of nucleic acid, and not ordinarily of protein. Mobilities may be calculated *exactly* only for the fastest *descending* boundary, and thus the mobilities reported for boundaries in the ascending limb cannot be used in rigorous considerations of particle valencies. They are nevertheless reliable enough to reveal that the major boundary migrates with a velocity higher than that characteristic of most proteins (Alberty, '53).

The nucleic acid present in component "2" must make a contribution to the electrophoretic mobility which is out of proportion to its relative mass, for the analytic data show that the major boundary is due to a component which quantitatively is mostly protein. The sedimenting particle therefore may well be a nucleoprotein particle, and not a randomly associate, polydisperse system of protein and nucleic acid. Electron micrographs are in support of this conclusion (Gross, '56).

The smallest and fastest peak of the control is absent from the experimental pattern. Although this phenomenon cannot be of quantitative significance in the overall events which follow calcification of an homogenate, its existence reveals the presence of a second Ca-sensitive component. Its very high mobility must be interpreted as due to an exceptionally high charge density, and this later suggests free nucleic acid or, perhaps, a polysulfuric ester polysaccharide, such as the one known to be present in the jelly coat of the *Arbacia* egg. Perhaps the small peak in the control pattern reveals incomplete removal of the jelly coat material in the processing prior to homogenization. It is known that the jelly coat material binds calcium (see, for example, Rudenberg, '53).

The failure of significant proportions of the added calcium to sediment with the extra material thrown down in the experimental systems of experiment 54III may provide a primary clue to the mechanism of the polymerization reaction; its extension and further confirmation is a task of some importance. This failure must be interpreted as follows: the aggregation reaction which leads to increased sedimentability of nucleoprotein is not a simple charge neutralization or reversal due to electrostatic binding between cation and anionic macromolecule, for were this the case, at least some Ca should have been lost from the water extracts in experiment 54III, and the amount lost should have increased, if only slightly, as the amount of insoluble nucleoprotein increased. That did not occur. A positive interpretation of the result might be that a reaction is initiated by small amounts of Ca which then proceeds over (in this case) a rather long time-course, and which results in a change in some macromolecular fractions but in no overall change in the Ca. Such a situation might result from the action of a Ca-activated enzyme upon the sensitive macromolecule, and in this connection the discovery of Ca-activated proteolytic activity in these homogenates by Lundblad ('54) and by Gross (see '54) is of interest. The existence of these proteases, considered in the light of the present results, makes it a distinct possibility that the aggregation reactions (and, by reasonable extrapolation, the cytoplasmic gelation) depend upon a kind of limited proteolysis. The recent observations of Ferry ('54) upon intermediate-polymer steps in the clotting of fibrinogen by thrombin are encouraging in this regard, for a primary test of any theory on the molecular basis of protoplasmic gelation is that it shall provide for ready reversibility of the reaction. It is now a possibility that limited proteolysis clotting systems may under certain conditions represent equilibria, and therefore the extension of information concerning the SPR to the cytoplasmic gelation process is not so highly speculative an effort as once it was.

Finally, it may be possible to assign, on the basis of these experiments, a new role to the small nucleoprotein particles (microsomes?) of cytoplasm, i.e., that of giving to cytoplasm its colloidal properties and controlling their alteration. On this basis might be explained, for example, the observations concerning changes in cytoplasmic basophilia following fertilization, quoted, together with an explanation at variance with the one here proposed, by Runnström ('51). Probably, it is just these small particles, rich in PNA, which provide the basophilia of the ground substance. It may be premature to propose it as a fact, but it is not too early to suggest as a hypothesis this physiological role for the particles in question. Current biological thought contains reference only to possible metabolic properties of these particles, such, for example, as a supposed participation in protein synthesis. The present hypothesis does not disclaim that function, or indeed any metabolic function; rather, it proposes for these nucleoprotein particles a role in a different domain, a domain, however, in which changes would markedly change the properties of the metabolic one. It is propsed largely with a view to the formulation of further experimental questions, which ask, specifically, whether or not there exists in these particles the molecular basis of those changes in the cytoplasm which so universally signal preparation for cell division, and response to stimulation.

SUMMARY

In a fraction of a Ca-free *Arbacia* egg homogenate which contains no pigment granules and little yolk, the addition of calcium ions in small quantities causes a nucleic acid-containing particle to lose its solubility in water and become sedimentable at 25,000 × g. The particle is largely protein, but its sedimentation results in a faster drop, percentagewise, in the extractable PNA than it does in extractable total protein. The added calcium ion, which initiates the "precipitation" reaction, remained soluble and unattached to the aggregating particles, as shown by titration with EDTA.

Electrophoretic analysis of water extracts of the homogenates reveals that the material which precipitates comes mainly from one component of the extract. This is further the main component in terms of concentration of particles, and it is characterized by an extremely high electrophoretic mobility.

The significance of these observations in terms of reaction mechanism, the SPR, and the cytoplasmic sol-gel transformation is discussed, and a hypothesis is made concerning a possible colloidal function of small nucleoprotein particles in cytoplasm.

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EXPERIMENTS UPON THE HEART OF CHAOBORUS ALBIPES (DIPTERA, CULICIDAE)

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TWO FIGURES

The larvae of *Chaoborus* (= *Corethra)* are among the most unusual of all insect larvae. Although studied by early microscopists (Lyonet, 1832; Réaumur, 1734; Slabber, 1778), our knowledge of their anatomy was not placed upon a sound basis until the studies of Jones (1867), Leydig (1851), and Weismann (1866). The structure of the heart has been most thoroughly studied by Dogiel (1877), Lebrun ('26), Leydig (1851) and Tzonis ('36).

Only Dogiel (1877) and Tzonis ('36) have studied cardiac physiology with any depth. Tzonis concerned himself primarily with anatomy as related to function, with the normal sequence of systole and diastole, and with the effect of temperature. Dogiel performed a number of physiological experiments, some of which will be mentioned in the body of the paper.

Our knowledge of cardiac physiology in other insects has been reviewed by Beard ('53), Nutting ('51), and Wigglesworth ('39, '50). Hearts of various insects differ considerably from one another. No agreement exists among authorities concerning the mechanism involved in the origin of the beat or the conduction of the impulse. It is hoped the results of the experiments described below will be a modest contribution towards our understanding of these matters.

MATERIALS AND METHODS

Most of the specimens of *Chaoborus albipes* were collected August 9, 1954 from the plankton of Cloverdale Lake, near Painesville, Ohio, and were maintained in wide-mouth gallon jars for the period of the investigation. A few of the specimens that were studied were collected on September 17, 1954 in the flooded basement of an abandoned garage near the Black River in Lorain County, Ohio. These were of another, unidentified, species of *Chaoborus*. Gratitude is acknowledged to Mr. William E. Kelley, Director of the Cleveland Aquarium, for information regarding the latter source of supply of *Chaoborus*.

With the exception of a few pupae, all the experimental animals were last-stage larvae. The majority of the experiments were performed either with the use of ligatures, or by cauterization, or both simultaneously. Ligatures consisted of fine winter hairs of a dog (diameter of hairs approximately 30 μ), tied with a simple half-hitch around the body. In no case was any evidence of leakage in or out across the ligature observed. Cauterization was performed according to the technique of Alverdes ('26). Larvae were gently lifted from the water by means of forceps whose points were constructed of the eyelashes of swine, dried on a small piece of filter paper, and then cauterized with a hot needle at the desired position. All cauterizations were performed under the magnification of a dissecting microscope. The needle was brought to a glowing temperature, but was carried a meter through the air before being touched to the larva, and was never glowing at the time of contact. Tissues affected by the heat were readily detected by their opacity and somewhat yellowish cast, in contrast to the hyaline healthy tissues. Observations were made either in a Syracuse watch glass, or on an ordinary glass slide under a cover slip. In the latter case, ordinarily sufficient water was provided to prevent any untoward effects from the pressure of the cover slip. In certain experiments, however, pressure from the cover was applied by allowing the ambient water to evaporate slowly. Temperatures were unregulated, so that observa-

tions and experiments were made at various ambient temperatures, between 19° and 31°C.

Heart rates were obtained, both before and after operation, by use of a hand tally and stop-watch. Most counts were made for a period of a full 60 seconds, but some were continued for longer or shorter periods, as appropriate.

The normal sequence in the beating of the heart

Dogiel (1877), Leydig (1851), and Tzonis ('36) described the sequence of events during cardiac action in *Chaoborus*. Contraction begins at the posterior tip of the heart, in the 8th

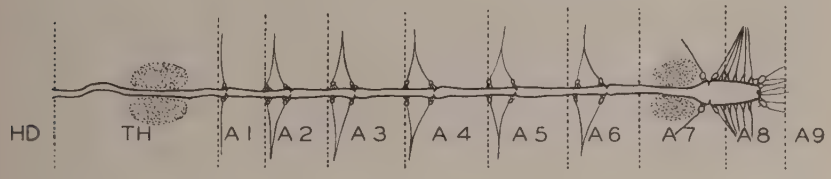

Fig. 1 The larval heart of *Chaoborus* sp., dorsal view. The vertical dotted lines indicate the position of the body sutures. The four oval stippled areas indicate the two pairs of tracheal air sacs. HD, head; TH, thorax; A1, A2, A3 . . . A9, 1st through 9th abdominal segments. The position of the pericardial cells and the aliform ligaments is shown.

abdominal segment, and passes forward in a distinct peristaltic wave. The passage is slow, so that at any one time there occur on the heart as a whole three systoles and two diastoles, or two systoles and three diastoles. Figure 1 shows the basic structural features of the heart.

Except at the posterior tip of the heart, where the additional diastole is lacking, the normal sequence of the beat is as previously described by Tzonis ('36): systole — pause — diastole — pause — additional diastole — systole — etc. If a graphic presentation of these events were available, the additional diastole apparently would correspond to the presystolic notch obtained (Yeager, '38) in mechanocardiograms of the roach heart. It is easily seen in the transparent larvae of *Chaoborus* that it is produced by the blood being pushed for-

ward by the approaching systolic wave. Yeager ('39) came to the same conclusion for the cockroach on the basis of results he obtained by the electrical stimulation of isolated segments of the heart.

Beard ('53) has tabulated the results of various authors who have studied the rate of the heart beat in various insects. The rate varies, of course, with the temperature. In *Chaoborus,* however, even at high temperatures it is slower than in most other forms. In the present study the rates in intact animals were counted at temperatures varying between 19°

TABLE 1

Rate of heart beat of intact larvae at different temperatures

TEMPERATURE	AVE. RATE	RANGE	NO. OF OBSERVATIONS
°C.			
19	16	..	1
20	17	16–19	3
21	17	..	1
22	17	17–18	3
23	21	..	1
24	25	21–31	3
25	22	21–25	3
26	27	24–30	2
27	24	19–29	7
29	32	..	1
31	34	32–37	2

and 31°C., as shown in table 1. They approximate the rates obtained by Dogiel (1877), Frankenberg ('15), Leydig (1851) and Tzonis ('36), though considerably higher results than ever previously reported were observed in some of the experimental animals.

Accelerator effect of the nervous system

The nerve supply to the heart of many insects is complex. Often there is a pair of lateral cardiac nerves running along the heart and sending branches to it, to the alary muscles, and to the lateral vessels. These nerves are composed primarily of fibers from the cardiac ganglia of the stomatogastric sys-

tem, but also of fibers from the ventral ganglia of the central nervous system, through the segmental cardiac nerves (Alexandrowicz, '26; McIndoo, '45). No cardiac innervation has been described for larval *Chaoborus,* with the exception of the unconfirmed, and probably mistaken, description given by Wagener in 1874 (see below). Among the Diptera, Weismann (1864) reported cardiac innervation in *Musca,* and Yaguzhinskaya ('54) demonstrated nerve endings, from segmental nerves, in the wall of the heart of the crane fly, *Pachyrrhina cornicina,* but neither she, nor Jones ('54) could trace segmental nerve endings to the heart in *Anopheles.*

The view of Dogiel (1877) and Dogiel and Archangelsky ('06) that the pericardial cells are "apolar nerve cells" is of historical interest only, as are the views of Jones (1864) and Akehurst ('22) that these cells are "ganglia" and that the alary ligaments are "short nerves." All recent authors assign other functions to pericardial cells and to alary ligaments.

It was observed that after disturbing the larvae the rate of the heart beat increased. As a representative example, the excitement associated with the placing of a ligature around the body of a particular larva resulted in an increase from 21 to 26 systoles/minute. Subsequently the rate dropped back to 19 systoles/minute when the animal was again at rest. In a second case a larva was very vigorously disturbed. The rate increased from 23 to 32 systoles/minute, whereas an undisturbed control maintained a constant rate of 25 systoles/minute. Similar results have been described by Dogiel (1877) and Tzonis ('36). Such findings suggest a nervous influence upon the heart rate, despite the lack of any demonstration in the literature of cardiac innervation in *Chaoborus.*

Point of initiation of the beat

The beat of the heart in healthy, intact larvae, always originates at the very posterior tip of the heart, as described above. In this respect, *Chaoborus* larvae do not differ from other larval insects. A number of experiments were undertaken to de-

termine whether this origin would be invariable under all conditions.

In every case where simple ligatures were applied around the body of larvae, the beat of the heart anterior to the ligature became irregular, with shorter or longer pauses between groups of rhythmic beats. Within the groups of beats, how-

TABLE 2

Results from simple ligature experiments

CASE NO.	LIGATURE POSITION	HEART RATES, SYSTOLES/MINUTE		
		Before operation	Posterior to lig. (ave.)	Anterior to lig. (ave.)
1	Between segs. 6 and 7	?	36	32, somewhat irreg.
2	Between segs. 6 and 7	19	21	Somewhat irreg., approx. old rate
3	Post. part of seg. 6	31	40	Irreg., approx. old rate
4	Between segs. 4 and 5	31	31	Irreg., approx. old rate
5	Between segs. 3 and 4	25	33	Very irreg., approx. old rate
6	Middle of seg. 3	22	29	Occasional systoles only
7	Between segs. 1 and 2	21	22	Irreg., approx. old rate
8	Between segs. 1 and 2	16	33	Very few systoles
9	Between thor. and seg. 1	17	19	None

ever, the rate usually remained approximately the same as before the operation. The rate and rhythmicity of the beat posterior to the ligature, on the other hand, was affected relatively slightly by simple ligatures. Results are shown in table 2.

Similarly, in transected larvae, regardless of whether or not the body was ligatured previous to the operation to prevent

the loss of blood, the heart anterior to the cut continued to beat more or less regularly. In these cases also, groups of beats occurred at approximately the same rate as the rate of the heart previous to the operation. Experimental results are shown in table 3.

It was found that the synchrony of the heart could be disrupted by cauterization, and a number of instructive results were observed, as detailed in table 4. In every case but one the heart continued to beat more or less regularly in abdominal segments anterior to the cautery. The origin of the beat occurred anywhere as far anterior as the 5th abdominal segment,

TABLE 3

Effects of transection upon the anterior heart rate

CASE NO.	LIGATURE POSITION	TRANSECTION POSITION	RATE ANTERIOR TO CUT (EST.)	PRE-OPERATION RATE
1	Between segments 5 and 6	Segment 6	Irreg., approx. old rate	32
2	Middle of segment 6	Segment 6	28, irreg.	21
3	None	Middle of seg. 3	20, irreg.	20
4	Middle of segment 3	Segment 4	Occasional beats only	22

and failed to occur only in an example where cautery was performed upon the 4th segment.

In those cases where the heart also continued in action posterior to the cautery, the beating in the two portions was never synchronous, and usually the two rates differed to a greater or lesser extent. In one experiment (table 4, case no. 6) the heart beat at 4 different rates, namely 39 systoles/minute originating at the posterior tip of the heart, 29 systoles/minute originating in the posterior portion of the 7th abdominal segment, irregular (and asynchronous) just posterior to the valve in the 6th abdominal segment, and 33 systoles/minute just anterior to the same valve.

TABLE 4

Results of posterior cauterization upon the point of origin of the beat of the heart

CASE NO.	CAUTERY LOCATION	POSITION OF HEART DESTRUCTION	ANT. POINT OF ORIGIN OF BEAT	RATE	REMARK
1	Segment 8	Post. part of 8th chamber	Ant. 1/3 of seg. 8	39	Entire heart sto 1st, then comme cept post. tip.
2	Segment 7	In segment 7	Segment 7, region of air sacs	34	Post. rate (39) chronous
3	Segment 4	Segment 4	None observed	..	Rhythmic beating iorly
4	Segment 8	Segment 8	Very ant. part of seg. 8	25	At 1st, origin in of seg. 7, then back
5	Segment 7	Segments 6, 7 and 8	Segment 5	Irreg. (11)	Stopped entirely again 6 min. af cautery
6	Ant. part of seg. 7	Segments 6 and 7	3 anterior origins: post. part of seg. 7, just post. to valve in seg. 6, and just ant. to valve in seg 6	29 in seg. 7; irreg. in post. of seg. 6; 33 in ant. of seg. 6	Rate at post. tip 8 is 39 systoles
7	Segment 7 and ant. part of seg. 8	Post. part of heart, segs. 7 and 8	Segment 6	Irreg. (27)	Entire heart sto 1st, never recove teriorly
8	Ant. 1/2 of seg. 7	Segment 7	Segment 6	23	25 systoles/minut 8; no contracti seg. 7
9	Segment 9	Near post. tip of heart, segment 8	Segment 8, near tip	37	Post. tip of heart at 1st did not be commenced asyn ously. 74 systol
10	Segments 8 and 9	Segment 8	Post. part, seg. 7	22	19½ hrs. after ca beat confined to
11	Between segs. 6 and 7, and segs. 8 and 9	Segments 6, 7 and 8	Ant. part of seg. 6; ant. part of seg. 8	18 and 14	Beat in segs. 8 a gan only 34 mi cautery. Post. heart never reco
12	Segment 6	Segment 6	Post. part, seg. 5	30	Posterior rate al but asynchrono

In case no. 9 (table 4) the anterior beat originated in the 8th segment very near the posterior tip of the heart, the rate being 37 systoles/minute. Another beat originated at the usual position at the very tip of the heart, with the extraordinary rate of 74 systoles/minute. At the end of 21 hours the posterior tip of this heart was still beating distinctly more rapidly than it was a few microns farther forward in the 8th segment.

From the above experiments, and from limited experimental results described by Dogiel (1877), it is evident that the origin of the beat is not necessarily always at the posterior tip of the heart in *Chaoborus,* but may be elsewhere when the posterior influence is removed by one means or another. As also has been shown by Dogiel (op. cit.), the dominance of all posterior influences (providing they can be conducted forward) indicates the occurrence of a physiological gradient that becomes weaker farther forward. Similar conclusions were reached by Jones ('54) for *Anopheles.* (Experimentally induced reversals in the most posterior chamber of the heart will be discussed below.)

Beard ('53) performed ligation experiments on the heart of larvae of *Galleria mellonella,* and found that the posterior portion beat regularly, but that the portion anterior to the ligature beat more slowly, either in or out of phase with the posterior. Ligature experiments with *Oncopeltus fasciatus* resulted in waves of cardiac contraction which commenced at both ends of the heart, and then moved anteriorly and posteriorly respectively, to meet at the ligature (they were not necessarily synchronous). In *Acheta* the portion anterior to the ligature beat forward, and that which was posterior beat alternately forward and backward. Hearts of larval and adult *Anopheles quadrimaculatus* were transected by Jones ('54). In transected larval hearts the anterior portions usually continued their rhythmic contractions, although at a reduced rate. Both Beard and Jones concluded that each abdominal segment of the heart potentially can initiate the beat, a conclusion fully supported by the results detailed above.

Reversal of the heartbeat

Normal reversal of the peristaltic wave of contraction in the heart of pupal and adult insects has been observed in several insect orders (Gerould, '29a, '29b, '33). Among the Diptera, Yaguzhinskaya ('54) described such reversals in *Anopheles maculipennis*. Normal reversals apparently have not been reported for larval stages.

In the present study a reversal was seen in four of the experimental larvae, but only in the 7th and/or 8th abdominal segments. These reversals never occurred except after a considerable period of stress, caused by blocking of the heart by cautery anterior to the 7th and 8th segments, or to pressure nearly to the point of death (exerted through the gradual evaporation of the ambient water from beneath a cover slip). Reversals did not necessarily indicate a moribund heart, for affected hearts continued beating strongly for as much as 16½ hours after the initiation of reverse peristalsis.

Ligature experiments by Beard ('53), discussed above, similarly suggest that conditions of stress initiated the onset of reverse peristalsis in the two insects he studied (*Oncopeltus fasciatus* and *Acheta* sp.).

The automatism of the heart

There has been considerable discussion of the automatism of the insect heart, with little agreement among authors. As has, with merit, been suggested by Beard ('53) and by Jones ('54), it is not necessary to assume that all insects are identical in the origin of the heart beat. The three main theories are (1) the distension theory; (2) the neurogenic theory, and (3) the myogenic theory.

1. The distension theory. In his studies of the heart of *Chironomus*, Dubuisson ('29, '30) concluded that the aliform ligaments are veritable muscles, the contraction of which stretches the heart muscle transversely and unlatches the peristaltic wave. The effect of distension has been reviewed by de Wilde ('48), who pointed out that this is the case only in

Chironomus, and that in other insects the alary muscles (or ligaments) either do not contract, or they contract asynchronously with the heart. Some authors, such as Duwez ('38), consider that the stretching of the heart is a factor altering the frequency of the automatism, but not determining the automatism itself.

In the heart of *Chaoborus* larvae the aliform ligaments are striking objects. In each of the abdominal segments of the body one pair is attached in the region of the ostia, and a second pair is attached somewhat anterior to this. Each ligament branches, and sends fine ramifications over the surface of the heart, as shown beautifully by Dogiel (1877). In the 7th and 8th abdominal segments there are numerous such branched fibers attached to the 8th chamber of the heart. Especially in the posterior chamber, the fibers can be seen to lengthen when the heart contracts, and to shorten during diastole. Among others, Dogiel (op. cit.) described these fibers as muscles, but both Lebrun ('26) and Tzonis ('36) could detect no evidence of intrinsic contraction, and considered them as elastic fibers. On the basis of repeated examinations of many intact and experimental animals the present author accepts this latter interpretation.

In the normal larvae of *Chaoborus,* except in the posterior portion of the posterior chamber, systole is always immediately preceded by a second diastole, produced by the flow of blood from behind. That this second diastole is unnecessary for systole is shown by the fact that the origin of the systolic wave at the posterior tip of the heart is not at this point immediately preceded by a diastole. Furthermore, in experimental hearts the contraction often originated in unusual portions of the heart without the benefit of any real diastole. For example, in an experiment already described above, a larva was ligatured between the 1st and 2nd abdominal segments and in the middle of the 6th segment, and the anterior and posterior portions of the body were cut away. The contraction of the heart originated at a point in the 6th segment where no trace of the 2nd diastole could be seen, and the 1st

diastole was extremely feeble. Other similar cases were observed where no distension was possible at the point of origin of the beat, whether from within by the flow of the blood from behind, or from without by the shortening of the aliform ligaments.

On the other hand, the results obtained from one experimental animal show that internal distension of the heart can stimulate systole. An early pre-pupa had been cauterized on the head, and on the dorsal surface between abdominal segments 6–7 and 8–9. The heart stopped permanently posteriorly, but there was no visible evidence of heart damage in the anterior part of the 6th segment. Within 10 minutes the anterior portion of the heart was beating consistently, although somewhat irregularly. The systolic wave clearly originated in the anterior portion of the 6th segment, half way between the valves and the border between segments 6 and 5. The peristaltic wave passed forward in a normal manner. When each wave reached the valve region of the 5th segment, however, some of the blood was forced backward in the heart, distending the heart a second time in the posterior portion of the 5th segment. With each distension, an extra systole occurred in the portion of the heart immediately affected, obviously stimulated by the preceding abnormal diastole (see fig. 2). Hence, at the position in question there were twice as many systoles as either behind in the 6th segment or forward in that portion of the heart lying anterior to the valves in the 5th segment.

It appears that in larval *Chaoborus,* unlike the situation in *Chironomus* described by Dubuisson ('29, '30), the function of the aliform ligaments is not to initiate the systolic wave by the distension of the wall of the heart. Furthermore, the additional systolic wave characteristic of the heart is not necessary for its automatism, though it probably helps in the normal sequence of diastole and systole. Perhaps it is at least one of the means of transmittal of the propulsatory wave of contraction.

2. The neurogenic theory. Carlson ('06), reasoning from his previous study of the heart of *Limulus,* tended to think

that insects possess a neurogenic heart. The fact that the μ value of many insect hearts is in the same range as has been observed in many invertebrate nervous systems, and in structures controlled by the nervous system, has led Fries ('27) and Jahn and Koel ('48) to the same conclusion, though the latter authors agree that the evidence is equivocal. Daven-

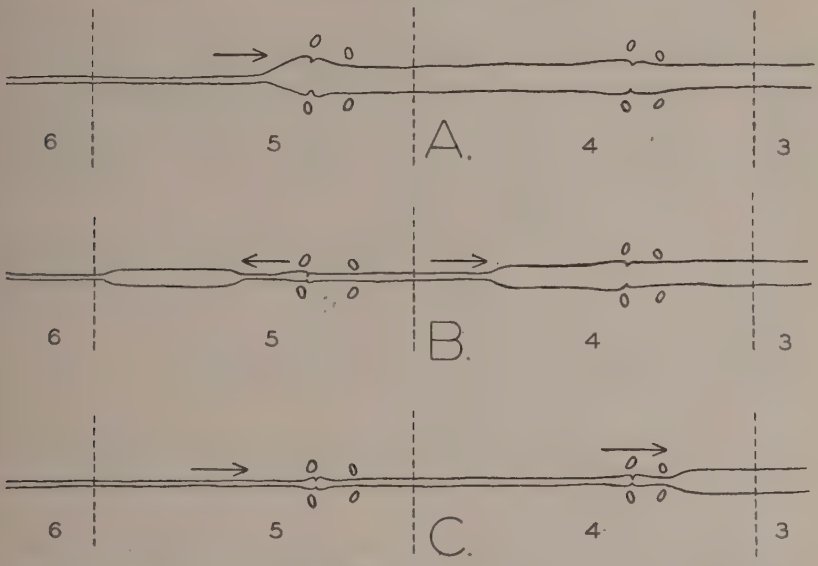

Fig. 2 Diagrams to show the effect of distension upon an experimental heart of *Chaoborus albipes*. Arrows indicate the direction of the flow of the blood. Numbers indicate the abdominal segments. *A:* The regular systolic wave, which originated in the 6th abdominal segment, is progressing forward in the normal manner. *B:* Some blood is abnormally forced backward from the valve region of the 5th segment, towards the posterior portion of the 5th segment, while the regular wave passes forward into the 4th segment. *C:* An extra systole occurs in the posterior portion of the 5th segment, forcing the blood forward, and apparently out through the valves of the 5th segment.

port ('49), Krijgsman and Krijgsman ('50), and Wixforth ('24) thought the heart was neurogenic, on the basis of pharmacological tests. Steiner ('32) believed that the heart was able to exhibit automaticity through the agency of ganglion cells that he demonstrated in the lateral cardiac nerves of the cockroach. On the other hand, Maloeuf ('35) and Zawarzin

('11) showed in the dragonfly and water bug that there are no ganglion cells in the lateral cardiac nerves, and that the heart continues to beat in the absence of all connections with ganglion cells. In reviewing the subject of the automaticity of the insect heart, Clark ('27) and Beard ('53) have felt that there is as yet insufficient evidence for the acceptance or rejection of the neurogenic theory, whereas Wigglesworth ('39) rejected the concept entirely. Recently, Jones ('54) and Yaguzhinskaya ('54) agree that the heart of *Anopheles* cannot be neurogenic.

In *Chaoborus* larvae, the only author who has described cardiac nerves connected with the central nervous system is Wagener (1874), in a brief statement in a paper primarily devoted to other matters. Wagener thought he saw a nerve derived from the brain, which ramified into several branches as it passed back over the heart, and whose endings very soon were lost to view. Inasmuch as no subsequent authors have described a similar structure, including those who studied anatomy of the heart in greatest detail (Dogiel, 1877; Lebrun, '26; Tzonis, '36), it may be assumed that Wagener mistook tracheae or connective tissue fibers for nerves.

As shown above in table 2, removal of the possible influence of anterior ganglia by tight ligatures had no particular effect upon the more posterior portions of the heart. Similarly, ligatures followed by transection did not essentially change either the rate or the amplitude.

In order to eliminate the possible influence of the posterior portions of the central nervous system remaining after transection, ventral ganglia of three transected larvae were destroyed by cauterization, as shown in table 5. In no case was the rhythmicity of the heart disrupted. From these experiments it is clear that the rhythmic beating of the heart in *Chaoborus albipes* is not dependent upon the ganglia of the central nervous system. Similiar conclusions have been reached for other insects by Sasse ('11).

Beyond the possible action of the central nervous system, the arrangement of the pericardial cells circumstantially sug-

gests that they might be the pacemakers of the heart, but all recent authors have considered that they have other functions. For example, Keilin ('17), Kowalevsky (1889), and Matsuki ('27) have thought that the pericardial cells of *Chaoborus* and of *Chironomus* respectively are excretory cells, helping to purify the blood. Lebrun ('26) has suggested both that they serve as weights in the maintenance of the tension of the aliform ligaments, and that they are endocrine glands. Arvy and Gabe ('54) believed they were acyclic endocrine glands in *Prosopistoma.*

Results obtained in the present study support the contention that the pericardial cells are not involved as pacemakers of

TABLE 5

Effect upon heart of cauterization of ganglia in transected larvae

CASE NO.	LIGATURE POSITION	TRANSECTION POSITION	GANGLIA CAUTERIZED	HEART RATES		
				Pre-operation	After transection	After cauterization
1	Between segs. 3 and 4	Seg. 3	In segs. 6 and 7	25	31	25
2	Between segs. 1 and 2	Seg. 1	In seg. 7	16	33	39
3	Between segs. 3 and 4	Seg. 3	In segs. 4, 5, 6, and 7	29	31	35

the heart in *Chaoborus.* Frequently, in those experimental hearts where the origin of the beat lay anterior to the tip of the heart, the beat originated at points distant from the pericardial cells, and apparently not supplied with ramifications of the aliform ligaments. Perhaps the most convincing evidence that the pacemakers are not the pericardial cells was obtained in the experiment described above, where the heart was beating at 4 different rates in 4 different regions. In this larva there were two independent beats in the 6th abdominal segment. One of these originated just anterior to the ostium, and the other just posterior to the same ostium. If the pericardial cells were the pacemakers, then both of these regions

would have been "serviced" by the same cell, lying very nearby in the meshwork of the aliform ligaments. It is unlikely that one cell could initiate two separate rates.

Under 100× magnification of a compound microscope, attempts were made to destroy individual pericardial cells. To this end, pressure was applied to larvae by means of the fine (diameter 50 μ) blunt tip of a glass rod drawn out in a flame. In only one case was the operation successful, at which time the right-hand large pericardial cell postero-lateral to the posterior tip of the heart was completely crushed. The heart continued to beat normally, the wave of contraction being initiated, as before, at the posterior tip of the heart on both the right and the left sides. If the pericardial cells were the pacemakers, the systolic wave should have originated only on the left side.

3. The myogenic theory. Some authors, such as Maloeuf ('35), Wigglesworth ('39) and de Wilde ('48), consider that the insect heart is myogenic. Beard ('53) and Clark ('27) also tended towards this belief, although they did not fully reject the possibility of a neurogenic origin of the beat, at least in some insects. Recently, Jones ('54) and Yaguzhinskaya ('54) have independently concluded that the heart of *Anopheles* is both myogenic and non-innervated.

The present results have led the author to accept the myogenic theory as the most likely explanation of the automaticity of the heart of larval *Chaoborus*. This conclusion was reached largely through the elimination of the other two theories, rather than upon direct supporting data. There is no evidence that the *Chaoborus* heart is innervated, other than the limited circumstantial evidence discussed above.

SUMMARY

1. The normal beating of the heart of *Chaoborus albipes* is as has been described previously: systole — pause — diastole — pause — additional diastole — systole — etc. The additional diastole is caused by the flow of blood from behind.

2. Stimulation or injury of larvae results in an increase of the rate of the heart beat, possibly through the central nervous system.

3. Ligature and transection experiments, and experiments where the larvae were cauterized dorsally to destroy portions of the heart, showed that the systolic wave can originate in any portion of the heart. Waves originating posteriorly ordinarily dominate over the more anterior portions if the impulse can be transmitted forward. There is, therefore, a physiological gradient, decreasing from behind forward.

4. When the impulse cannot be transmitted forward, the posterior and anterior portions of the heart beat asynchronously, and usually at somewhat different rates. In one experimental heart there were 4 different rates originating at 4 different positions.

5. Reversal of the heart beat was not observed in normal larvae, but occurred occasionally in the last chamber of the heart when the heart was blocked anteriorly, or was previously subjected to prolonged pressure.

6. The automatism of the heart is not thought to be the result of distension of the heart from within or from without. Instances are cited of experimental hearts where the systolic wave originated without any visible expansion of the walls. The aliform ligaments appear not to be the initiators of the systolic wave.

7. The pericardial cells appear not to be the pacemakers. In experimental animals the systolic wave often originated far from any pericadial cells. In one case the heart was beating at two different rates at points "serviced" by the same pericardial cell. Destruction by crushing of one of the two large pericardial cells at the posterior end of the heart did not alter the automaticity in any way.

8. Destruction by transection and cauterization of all parts of the central nervous system did not destroy the automaticity of the heart. The neurogenic theory does not appear to apply to larval *Chaoborus*.

9. The myogenic theory is considered the most likely explanation of automaticity in the heart of larval *Chaoborus*. Direct supporting evidence for this concept is sparse, however, and the conclusion was reached largely by the elimination of other explanations.

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BIOCHEMICAL AND PHYSIOLOGICAL DIFFERENTIATION DURING MORPHOGENESIS

XX. IN VITRO OBSERVATIONS ON CARBOHYDRATE METABOLISM OF THE DEVELOPING CEREBRAL CORTEX OF THE FETAL GUINEA PIG [1]

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FIVE FIGURES

A substantial body of information is now at hand on morphological, functional and biochemical changes which occur during the development of the frontal cerebral cortex of the fetal guinea pig (Flexner, '52). In particular these observations have been focused upon events which occur from the 41st to 46th days of gestation when neuroblasts differentiate into cells with the characteristics of immature neurons. With this background we have been interested in correlating changes in the metabolism of glucose with other events which occur during the maturation of the frontal cerebral cortex of the guinea pig.

METHODS

Pregnant guinea pigs were sacrificed by dislocation of the cervical vertebrae. Gestation age was estimated from crown-rump lengths of the fetuses (Draper, '20). The brain was rapidly removed from the skull, usually within two minutes of the

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death of the animal. So far as possible frontal cerebral cortex only was used. The tissue was weighed on a torsion balance and placed in buffered glucose as rapidly as possible. Undue delay in transferring the tissue to glucose leads to loss of glycolytic activity. In most instances the first sample was placed in glucose within 5 minutes after death of the animal and the last sample within the next 10 minutes. No difference in activity was noted between first and last samples. Minced material was routinely used.

Oxygen consumption only was measured in some of the experiments. In these the Warburg vessels contained 80 to 100 mg cortex, 2.4 ml Krebs ('33) Ringer-phosphate without calcium at pH 7.4 (Elliott, '48) glucose being 0.01 M; 0.3 ml of 20% NaOH and a wick were present in the center well. The vessels were gassed with CO_2-free O_2 and maintained at 37.5°. In other experiments rate of utilization of glucose and O_2 as well as production of lactate and CO_2 were measured. Glucose uniformly tagged with C^{14} was used in these experiments, and with respect to specific activity was compared with CO_2 produced. Because of irregular and confusing results obtained with the usual reducing methods as well as a method depending upon use of glucose oxidase and catalase, radioglucose was used to obtain the rate of glucose utilization. The Warburg vessels contained 80 mg cortex, 1.87 ml Krebs Ringer-phosphate without calcium, 0.005 M glucose of approximately 200 counts per minute per milligram glucose, 0.63 ml 1.25 N trichloroacetic acid in the center well and 0.20 ml 1.0 N NaOH, CO_2-free, in the side arm. The vessels were gassed with CO_2-free oxygen. The experiment was terminated by pouring of the trichloroacetic acid from the center well into the main compartment, giving a final concentration of 5% trichloroacetic acid. The NaOH in the side arm was next poured into the main compartment and measurement made of the increment of gas pressure, due to liberated CO_2. To recapture CO_2, 0.2 ml of 5 N NaOH, CO_2-free, was then quickly introduced into the side arm through its vented stopper without removal of the manometer from the bath. After reabsorption

of CO_2 the contents of the side arm were quantitatively removed and added to saturated $Ba(OH)_2$. The precipitated $BaCO_3$ was washed, dried, plated, weighed, counted and the counts corrected for absorption. An aliquot of the reaction mixture was analyzed for lactate by the Barker-Summerson ('41) method. Another aliquot was taken for radioactive glucose. The glucose was separated from contaminants by passing the solution through a column of anion exchange resin, Dowex 2-X10, in the acetate form. The glucose was demonstrated to be free of detectable radioactive contaminants after this treatment by paper chromatography, tests for lactate and by passing it through a column of cation exchange resin, Dowex 50-X8, in the acid form. The radioglucose was then counted and the total amount of glucose present determined from the known specific activity. In these experiments 0.005 M glucose was substituted for 0.01 M to give greater sensitivity in the radioactivity measurements after the observation that the rate of utilization of glucose was the same with the two concentrations. Five manometers were used in each of these experiments. In one the reaction was stopped by addition of trichloroacetic acid to the main compartment after the gassing and temperature equilibration, usually 20 minutes after tissue was placed in the buffered glucose. The reaction was stopped in the remaining manometers at 50, 80, 110 and 140 minutes, respectively, after the tissue was placed in buffered glucose. To test the effect of the nature of the buffer on rates of glucose utilization and lactate production another series of experiments were run with Krebs bicarbonate buffer and a gas phase of 95% O_2 and 5% CO_2. These experiments were identical with those just described except that trichloroacetic acid and NaOH were not present in the flasks and the volume of the reaction mixture was correspondingly increased. A series of observations were made on rate of aerobic glycolysis alone with the use of 0.01 M glucose in Krebs Ringer-bicarbonate and a gas phase of 95% O_2 and 5% CO_2.

Two series of experiments were made under anaerobic conditions. In the larger series only rate of accumulation of lac-

tate was measured; in the smaller, rate of disappearance of glucose, with uniformly tagged material, was also measured. The Krebs Ringer-bicarbonate buffer with 0.01 M glucose was gassed with 95% nitrogen and 5% CO_2. In a few experiments, to test the possibility that phosphate might limit the rate of anaerobic glycolysis, supplemental phosphate was added. The volume of solution, amount of tissue and temperature were as in the aerobic experiments. The rate of formation of lactate was obtained from increase in CO_2 pressure and invariably

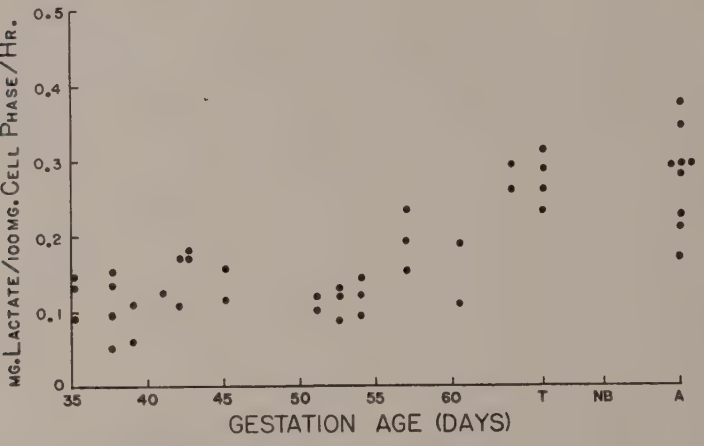

Fig. 1 Rate of aerobic glycolysis of frontal cerebral cortex as related to developmental age. T = term; NB = newborn; A = adult.

checked by the Barker-Summerson method. Results are expressed in terms of a unit weight of cell phase (Flexner and Flexner, '49).

RESULTS

Aerobic glycolysis. Rate of aerobic lactate production in glucose Ringer-bicarbonate was found to be proportional to the amount of tissue present and was not affected by additional inorganic phosphate. As shown in figure 2, there was a relatively rapid rate of production of lactate during the first 20 minutes in glucose-phosphate or bicarbonate followed by a slower rate of accumulation which was linear for at least one

hour. The initial, rapid rate of production of lactate was correlated with an increased rate of disappearance of glucose (fig. 2) which probably was related to the deprivation of glucose during preparation of the tissue. For this reason the values of figure 1 obtained in glucose-bicarbonate represent the rate as observed after the first 20 minutes, i.e., the lactate present in control vessels at the end of 20 minutes was subtracted from that present in vessels maintained for a longer

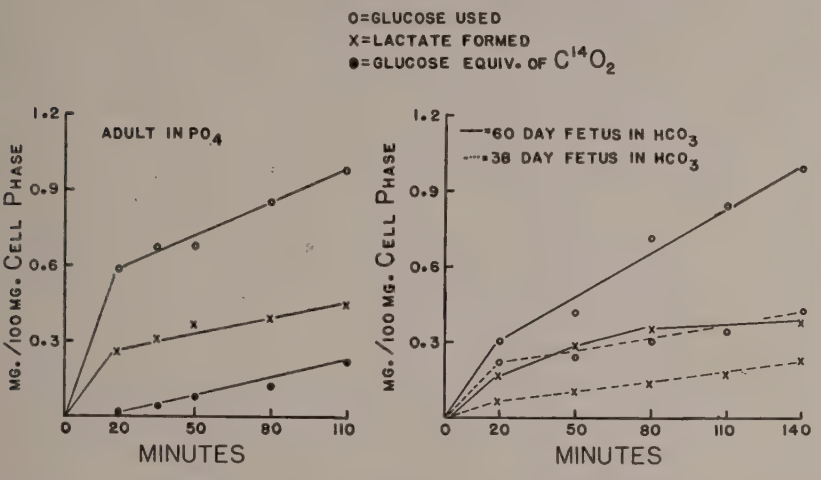

Fig. 2 *Left.* Rates of glucose disappearance, lactate production and $C^{14}O_2$ formation (in terms of its glucose equivalent) of adult frontal cerebral cortex in phosphate-Ringer and an atmosphere of O_2. The glucose was uniformly labelled with C^{14}. *Right.* Rates of glucose disappearance and lactate production with the cerebral cortex of 38 and 60 day fetuses in bicarbonate-Ringer and an atmosphere of 95% O_2 and 5% CO_2. The glucose was uniformly labelled with C^{14}.

period. The rate of formation of lactate given in figure 1, calculated on the basis of a unit weight of cell phase, shows considerable scatter with an indication of approximately a 2-fold increase at term and in the adult, the increase beginning between the 57th and 64th days of gestation.

Aerobic rate of glucose disappearance. The observations with radioglucose were largely limited with respect to each buffer used to single samples of cerebral cortex at three stages of development: (1) before the period of differentiation of

neuroblasts into immature neurons at the 41st to 46th days, (2) after this period, and (3) in the adult. Figure 2 gives several typical experiments in phosphate and bicarbonate buffer. In all instances there was an initial relatively rapid rate of glucose uptake which ended within 20 minutes after placing the tissue in the glucose medium. The older the cortex, the greater was this initial rate in both buffers (table 1). The initial rapid rate was followed by a slower rate of disappearance which continued linearly for the next hour and then occasionally declined in the following hour. Again, the older the cortex, the greater the rate of glucose disappearance in this second period with one exception in phosphate buffer (table 1). The differences in bicarbonate were more striking than in phosphate; rates of glucose disappearance increased 4-fold from the neuroblast stage to the adult in bicarbonate but only 2-fold over the same developmental period in phosphate. In both buffers, the substantial portion of the change percentagewise in rate of glucose disappearance occurred between the early stages and term.

Rate of glucose oxidation. The amount of glucose oxidized was calculated from the radioactivity of the CO_2 collected in the experiments with phosphate buffer. In all experiments, there was a linear rate of glucose oxidation lasting for at least 90 minutes after the initial 20-minute period during which the flasks were gassed and equilibrated (fig. 2). Within the error of the experiments, the glucose which disappeared after the first 20 minutes could be accounted for by the CO_2 derived from it and the lactate formed (table 1). Accordingly, as an approximation, in the experiments with bicarbonate buffer rate of glucose oxidation was obtained from the difference between rate of glucose disappearance and of lactate formation.

The results in both buffers show an increase in rate of glucose oxidation with development amounting to a 16-fold increase in bicarbonate and a 4-fold increase in phosphate Ringer (table 1). In both buffers, the substantial portion of the change percentagewise occurred between the early stages and term.

Observations on rates of glucose uptake and oxidation in bicarbonate and phosphate Ringer as well as O_2-consumption, O_2-equivalent of glucose oxidized, ratio of specific activity (S.A.) of carbon of CO_2 produced to that of glucose present and respiratory quotients (R.Q.) in phosphate Ringer. All rates are per 100 mg cell phase. The rate of oxidation of glucose in PO_4 (1) is the difference between the glucose which disappeared and the lactate produced; in PO_4 (2), rate of oxidation of glucose has been calculated from the radioactivity of the CO_2 produced.

FETAL AGE	INITIAL RATE OF GLUCOSE USE IN		SECOND RATE OF GLUCOSE USE IN		RATE GLUCOSE OXIDATION IN			O_2-UPTAKE IN PO_4	O_2 EQUIV. OF GLUCOSE OXIDIZED IN PO_4	S.A. CO_2 ÷ S.A. GLUCOSE × 100	R.Q.
	HCO_3	PO_4	HCO_3	PO_4	HCO_3	PO_4 (1)	PO_4 (2)				
days	*mg/hr*	*mg/hr*	*mg/hr*	*mg/hr*	*mg/hr*	*mg/hr*	*mg/hr*	*cc/hr*	*cc/hr*	%	
38	0.60	0.52	0.12	0.15	0.02	0.04	0.03	94	22	42	0.95
46		0.96		0.20		0.10	0.09	156	67	70	0.80
60	0.90		0.36		0.18						
66 (term)		1.2		0.34		0.15	0.16	194	120	48	0.85
adult	1.0	1.6	0.53	0.30	0.32	0.14	0.13	212	97	78	0.91

TABLE 2

Approximate energy derived from glycolysis and oxidation of glucose by the cerebral cortex at different stages of development. All values are per 100 mg cell phase. Net gain of ATP on basis of 2 moles of ATP per mole of glucose glycolyzed and 30 moles of ATP per mole of glucose oxidized (Ochoa, '41; Abood and Gerard, '52; Fruton and Simmonds, '53).

FETAL AGE	LACTATE FORMED IN		ATP FROM GLYCOLYSIS IN		GLUCOSE OXIDIZED IN		ATP FROM OXID. GLUCOSE		TOTAL ATP	
	HCO_3	PO_4	HCO_3	PO_4	HCO_3	PO_4	HCO_3	PO_4	HCO_3	PO_4
days	*μM/hr*	*μM/hr*	*μM/hr*	*μM/hr*	*μM/hr*	*μM/hr*	*μM/hr*	*μM/hr*	*μM/hr*	*μM/hr*
38	1.1	1.3	1.1	1.3	0.11	0.17	3.3	5.1	4.4	6.4
46		1.2		1.2		0.50		15		16
60	2.0		2.0		1.0		30		32	
66 (term)		2.0		2.0		0.89		27		29
adult	2.3	1.9	2.3	1.9	1.8	0.72	54	22	56	24

Oxygen consumption. The O_2-uptake in glucose Ringer-phosphate in milliliters per milligram cell phase per hour and as a function of developmental age is given in figure 3. Up to a gestation age of about 40 days, O_2-uptake was at a relatively low and constant level; it then began to increase and reached a value twice as great at term. There was a further increment in rate of uptake in the adult.

In the experiments with Ringer-phosphate and tagged glucose (table 1) the quantity of glucose oxidized as derived from

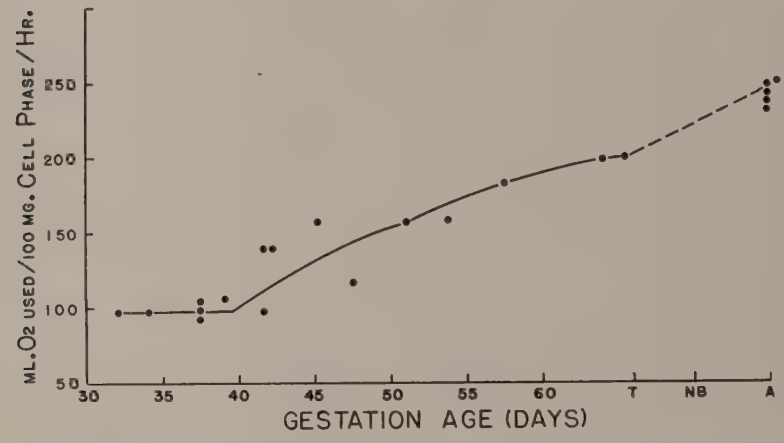

Fig. 3 Rate of O_2-consumption of frontal cerebral cortex in glucose phosphate-Ringer without calcium as related to developmental age.

the radioactivity of the CO_2 produced was considerably less than the glucose equivalent of the O_2 consumed; indeed, among the several experiments, cited in the table, only 25 to 60% of the O_2 consumed could be accounted for by the glucose oxidized.

Specific activity of CO_2; R. Q. Calculation of the specific activity of the carbon of the CO_2 produced in a vessel depends upon subtraction of the radioactivity of the CO_2 present at zero time in a control vessel after gassing and equilibration. Similarly, calculation of the respiratory quotient depends upon subtraction of the amount of CO_2 present at zero time. These values are not necessarily constant from one vessel to

another so that the corrections introduce a source of uncertainty. The values of table 1 have been obtained at the end of the period during which rates of glucose uptake, lactate production, O_2-uptake and $C^{14}O_2$ production are all maintained at a constant level and before the terminal decrease in one or more of these quantities sometimes observed. As shown in the table, the specific activity of the carbon of the CO_2 produced was regularly less than that of the carbon of the glucose used indicating, as was true with O_2-consumption, that substrates other than glucose were being oxidized. There is the possibility that the specific activity of the CO_2 produced was reduced by CO_2 derived from untagged glucose or carbohydrate intermediates present in the tissue. This does not appear to have been true, however, since no glucose was demonstrable in tissue after a period of time corresponding to time after gassing and equilibration and since the specific activity of the CO_2 did not increase with increased time of exposure to labelled glucose. The deduction that substrates other than glucose were oxidized is also to be reached, though in one instance less clearly, from the respiratory quotients. Both of these series of measurements have only qualitative meaning.

Anaerobic glycolysis. The rate of anaerobic lactate production in glucose Ringer-bicarbonate was found to be proportional to the amount of tissue present. As shown in figure 4 the rate of lactate production was linear for at least 140 minutes. The initial rate of disappearance of glucose was relatively rapid for the first 20 minutes after the tissue was placed in the medium. This initial rapid rate was followed by a slower rate of disappearance of glucose which was linear for at least two hours during which it had a slope indistinguishable from that of the rate of accumulation of lactate. This means that after the first 20 minutes the lactate produced was quantitatively accounted for by the glucose which disappeared and it appears therefore that the lactate was entirely derived from glucose. These observations were made in three experiments with radioglucose and with cerebral cortex from fetuses at the 35th and 49th days of gestation and from the adult.

As shown in figure 5, the rate of anaerobic glycolysis per unit weight of cell phase was relatively low up to about the 41st day of gestation and then sharply increased to reach at

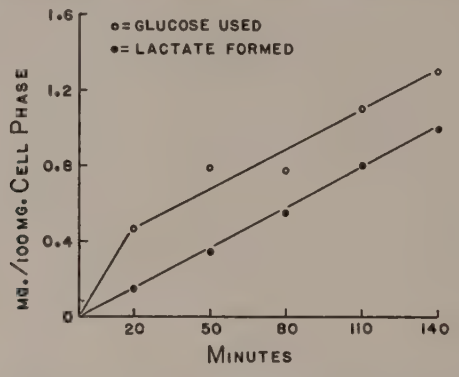

Fig. 4 Rates of anaerobic glucose disappearance and lactate formation with cerebral cortex from a 35 day fetus in bicarbonate-Ringer and an atmosphere of 95% N_2 and 5% CO_2. The glucose was uniformly labelled with C^{14}.

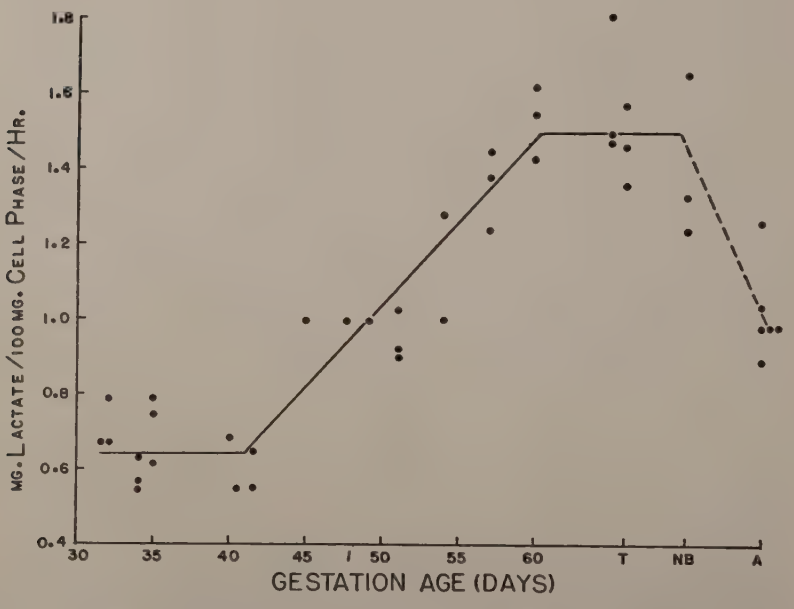

Fig. 5 Rates of anaerobic glycolysis of frontal cerebral cortex as related to developmental age.

the 60th day a value 2- or 3-fold greater which was maintained until term. The average rate in the adult was 35% lower than at term.

The values of figure 5 and of table 1 can be used to gauge the magnitude in bicarbonate-Ringer of the Pasteur effect and its relationship to the stages of development. The ratio of anaerobic to aerobic rate of glucose disappearance at the 38th day was 5; at the 60th day, 4; and in the adult, 2. Such considerations have the weakness that anaerobic and aerobic rates were not observed on the same cortices and that the aerobic rates were limited to a single example at each age.

DISCUSSION

Earlier studies (Flexner, Belknap and Flexner, '53; Flexner, Greenblatt, Cooperband and Flexner, '56) on the frontal cerebral cortex of the guinea pig have shown that the activities of the respiratory enzymes, succinic dehydrogenase and cytochrome oxidase, and the glycolytic enzyme, aldolase, are at a relatively low level of activity before morphological and functional differentiation occur and at the period of differentiation (gestation age 41–46 days) begin rather sharply to increase to that level characteristic of later fetal stages and the adult. If these assays of enzyme activity have functional meaning and are typical of enzymes involved in rate-limiting reactions, it might be predicted that maturation of the cortex up to term will be accompanied by increase in rates of utilization and oxidation of glucose and that the values at term will be much like those of the adult. These predictions have been largely substantiated by the results obtained in both phosphate and bicarbonate Ringer with C^{14}-glucose uniformly labelled. The immature neuron has a greater rate of both glucose uptake and glucose oxidation than the neuroblast and at the time of birth appears, with respect to these characteristics, much like the adult. Tyler and van Harreveld ('42) have observed less pronounced changes of the same kind in rate of

glucose used during postnatal maturation of the brain of the rat.

On current concepts, the amount of "high energy" phosphate as adenosinetriphosphate (ADP → ATP) which can be assumed to be formed from glycolysis and oxidation of glucose is given in table 2. It would appear that the cortical neuroblast obtains a considerable part of its energy from glycolysis. Maturation is accompanied by a doubling of energy derived from this source but, more importantly, by a substantial gain from oxidation of glucose. The total "high energy" phosphate made available to the neuroblast appears to be relatively low; more active synthesis of neuroplasm in the immature neuron as evidenced by the sprouting and growth of processes (Peters and Flexner, '50) and onset of functional activity (Flexner, Tyler and Gallant, '50) is accompanied by a progressive increase in the yield of ATP. There does not appear to be a marked decrease when the adult stage has been reached and growth has ceased; the energy available for growth and restricted function in the fetus is, on the basis of these *in vitro* experiments, essentially maintained for function in the adult. These findings are consistent with those obtained on other animals (Himwich, '51).

As has been observed in other species (Himwich, '51) maturation of the cerebral cortex of the guinea pig is accompanied by an increase in rate of O_2-consumption. This increase in the guinea pig begins at the period of differentiation of neuroblasts into immature neurons, and is correlated with an increase in activity of respiratory enzymes (Flexner, Belknap and Flexner, '53). The extrapolation of these *in vitro* values to the cortex of the intact animal is, however, highly questionable. The uncertainty arises from the evidence that only from 25 to 60% of the oxygen used in the several experiments can be accounted for on the basis of the glucose oxidized. In addition may be cited the relatively low specific activity of the CO_2 produced and the low values of the respiratory quotients. Sutherland, Burbridge and Elliott ('55) have also reported

an inconsistency between $C^{14}O_2$ derived from labelled glucose and oxygen consumption *in vitro* with human cerebral cortex.

The results on anaerobic glycolysis are of interest from two points of view. In the first place, Warburg, Posner and Negelein ('24) have made the generalization that rapidly growing tissues have high rates of anaerobic glycolysis. The rate in the neuroblast is low; with onset and maintenance of increased synthetic activity, as evidenced by the elaboration of processes (Peters and Flexner, '50), the rate increases and then declines in the adult. Much the same picture has been observed in the rat (Chesler and Himwich, '44). Secondly, the relatively high rates of anaerobic glycolysis near the end of gestation may have functional meaning in view of the low O_2-tension to which the brain of the living fetus may be exposed at this time (Barcroft, '47).

SUMMARY

A series of *in vitro* observations have been made on carbohydrate metabolism in the developing cerebral cortex of the guinea pig. Rate of aerobic glycolysis begins to increase late in gestation to reach values at term and in the adult twice that of earlier stages. Rate of glucose disappearance, with glucose uniformly labelled with C^{14} as substrate, increases 4-fold from the neuroblast stage to the adult in bicarbonate but only 2-fold over the same developmental period in phosphate. Rate of glucose oxidation in both buffers increases markedly with development. Rate of O_2-uptake begins to increase at the time of differentiation of neuroblasts into immature neurons and reaches a value twice as great at term as in the neuroblast. Among the several experiments only 25 to 60% of the O_2 consumed could be accounted for by the glucose oxidized. The specific activity of the CO_2 produced and the respiratory quotients also indicate that substrates other than glucose were oxidized. Rate of anaerobic glycolysis is low in the neuroblast; with increased synthetic activity of the immature neuron, the rate increases and then declines in the adult.

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DRY WEIGHT LOSS DURING CULMINATION OF THE SLIME MOLD, DICTYOSTELIUM DISCOIDEUM [1,2]

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In conducting some analyses of total reducing substances relative to the dry weight of the slime mold, *Dictyostelium discoideum*, it was found that the total reducing substances per dry weight increased by 44.2% during the transition from the vegetative stage to the mature sorocarp (Gregg and Bronsweig, '56). This was surprising since the slime mold amoebae cease feeding at the onset of aggregation and require no exogenous food supply during their morphogenetic processes. Major nitrogen changes as well as changes in total reducing substances occur during the culmination process (Gregg *et al.*, '54). The values of the reducing substances and nitrogenous components were based on the reference of dry weight. If a loss of dry weight occurred during the culmination of the slime mold, the values of reducing substances and nitrogenous components would appear greater than if the results could be expressed per *pseudoplasmodium* (it is impossible to express the results per pseudoplasmodium owing to the great variability in size of the individuals).

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It was of interest, therefore, to investigate the possibility that a loss of dry weight occurs in the slime mold, *D. discoideum,* during culmination. Such information would be of value in the interpretation of data based on a reference of dry weight.

METHODS

The slime molds were cultured according to Bonner's method ('47). However, the nutrient agar contained, in addition to the usual ingredients, 5.6 μc of P^{32}/ml of solid agar. The collecting procedure for migrating pseudoplasmodia and mature sorocarps was identical to the method used by Gregg and Bronsweig ('56) in that the slime molds were allowed to migrate from a nutrient agar surface to a non-nutrient agar surface containing Bonner's "standard solution" (Bonner, '47). Approximately 48–72 hours had elapsed from the inoculation of the plate with slime mold spores to the migration of pseudoplasmodia on the non-nutrient agar surface.

After the migrating pseudoplasmodia had reached the non-nutrient agar surface, some of them were collected by means of a hair loop and placed on tared discs of collodion film. Other migrating pseudoplasmodia were allowed to remain until fruiting had occurred. These mature sorocarps were then collected by means of fine-tipped forceps and placed on tared discs of collodion film. Following a 24-hour drying period at 60°C., all the samples were weighed to the nearest microgram on a quartz helix balance. Counts of migrating pseudoplasmodia and mature sorocarps containing P^{32} were made with a Geiger counter.

In determining whether the dry weight per unit mass of spores differed from that of the stalks, the vegetative amoebae were reared on nutrient agar plates which contained 21.0 μc of P^{32}/ml of solid agar. This concentration was found to be greater than necessary to obtain a count in *D. discoideum* and, therefore, the P^{32} content of the agar was reduced to 5.6 μc/ml of solid agar in subsequent experiments. When the mature sorocarps appeared, the spores and stalks were separated in

equal numbers by means of fine-tipped forceps and placed on small bits of cigarette paper. Counts of each were made with a Geiger counter.

RESULTS AND DISCUSSION

During the growth phase, the vegetative amoebae accumulated P^{32} probably by both absorption from the agar surface and by the ingestion of *Escherichia coli*. The assumption was made that populations of amoebae aggregating and forming migrating pseudoplasmodia will show an insignificant variation per unit mass in P^{32} content. Similarly, the variation in P^{32} content between pseudoplasmodia per unit mass will be negligible. In actuality, the P^{32} content is a relative index to the number of cells present in a pseudoplasmodium. Furthermore, the P^{32} content tends to remain constant during culmination with the exception of that lost through decay. P^{32} counts of migrating pseudoplasmodia and mature sorocarps from each experiment were made simultaneously. Thus P^{32} decay had occurred equally in each stage by the time the counts were made. The decay rate is therefore a factor which need not be considered in view of the design of the experiments. With a reference such as P^{32} it is then possible to determine if changes in dry weight occur during culmination.

The results illustrate that the average counts per minute per microgram of dry weight in the mature sorocarps is 6.6% higher than that of the migrating pseudoplasmodia (table 1). Therefore, during the transition from the migrating pseudoplasmodia to the mature sorocarps, a 6.6% loss in dry weight occurs. This difference is statistically significant ($P = < 0.05$).[3]

Since a loss in weight occurs during culmination, it was of interest to determine whether this loss occurred in the spores or in the stalks, or in both. It was assumed that no transfer of P^{32} occurred between spore and stalk cells during this process. By reference to table 2 the percentage of spore cells

[3] Statistical significance determined by the method of paired comparisons.

or stalk cells relative to a whole mature sorcarp may be determined. The relative proportions of the spores and stalks determined by P^{32} counts and the proportions determined by measurements of dry weight show very small differences. Therefore, it is suggested that the spores and stalks lose weight in approximately equal amounts.

TABLE 1

The loss in dry weight which occurs during the transition of slime molds from the migrating pseudoplasmodia to the mature sorocarps

EXP. NO.	STAGE IN DEVELOPMENT	(CTS/MIN)/μG OF DRY WEIGHT	AV. (CTS/MIN)/μG OF DRY WEIGHT	WEIGHT LOSS %
I	Migrating pseudoplasmodia	10.2 11.7	11.0	
	Mature sorocarps	10.2 12.8	11.5	4.3
II	Migrating pseudoplasmodia	9.85 9.50	9.68	
	Mature sorocarps	10.6 10.8 10.2	10.5	8.6
III	Migrating pseudoplasmodia	15.7	15.7	
	Mature sorocarps	18.1	18.1	13.2
IV	Migrating pseudoplasmodia	13.5 12.8	13.2	
	Mature sorocarps	13.6 12.9 14.1	13.5	3.0
V	Migrating pseudoplasmodia	11.4	11.4	
	Mature sorocarps	12.3 13.1	12.7	10.2
VI	Migrating pseudoplasmodia	14.8 15.9 16.5	15.7	0
	Mature sorocarps	15.7 15.6	15.7	

Av. loss in dry weight = 6.6%

Knowledge of the change in dry weight which occurs during the culmination process in *D. discoideum* prevents possible misinterpretation of data based on the reference of dry weight. This information may also be correlated with other data concerning the metabolism of *D. discoideum* during morphogenesis.

TABLE 2

The proportions of spores and stalks in the mature sorocarp determined by two methods

	CTS/MIN	NO. OF EXP.	DRY WEIGHT	NO. OF EXP.
Spores	20,306	6	858.8	11
Stalks	7,383	6	348.5	11
Total spores and stalks	27,689		1207.3	
Percentage of spores	73.3		71.1	
Percentage of stalks	26.7		28.9	

SUMMARY

1. A method of culturing the slime mold, *Dictyostelium discoideum,* in the presence of radioactive P^{32} was described.

2. The slime mold was shown to lose 6.6% of its dry weight during the transition from the migrating pseudoplasmodia to the nature sorocarp.

3. The loss in dry weight occurred from both spores and stalks in approximately equal amounts.

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COMMENTS AND COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

ON THE SIMILARITY OF RESPONSE OF MUSCLE TISSUE AND OF LAMPYRID LIGHT ORGANS [1]

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ONE FIGURE

The luminiscent response to stimulation of small pieces of *Mnemiopsis leidyi* tissue exhibits striking resemblances to certain aspects of muscle response (Chang, '54). The staircase phenomenon or "treppe" and summation of flashes result when the luminous tissue is stimulated at certain frequencies. So close are resemblances between the response characteristics of these two systems that the question has

[1] This research was supported in part by funds of the Eugene Higgins Trust allocated to Princeton University.

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been raised whether muscular activity could account for the photogenic responses of Mnemiopsis. A consideration of this possibility in the aforementioned work led to the conclusion that association of muscle fibers with the luminous cells of ctenophores seemed most unlikely.

Experiments have recently been carried out to observe the flash responses of two species of adult firefly, *Photuris pennsylvanica, Photinus pyralis* and the larval form, a glow-worm. In all these insects no muscle fibers are associated with the photogenic organ (McDermott and Crane, '11; Williams, '16;

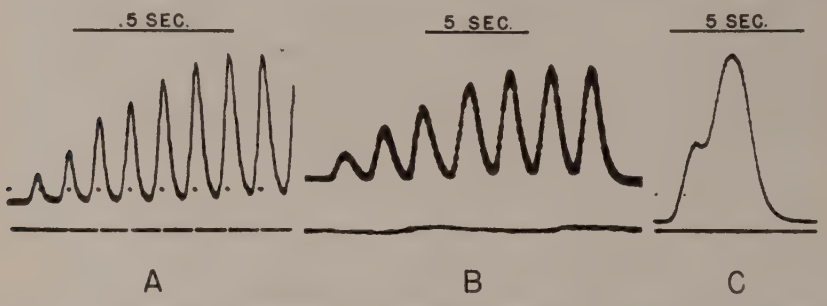

Fig. 1 Oscillograph recordings of luminous responses. Vertical axis represent light intensity, the horizontal the time.

Hess, '22; Buck, '48), but nevertheless the stair-case phenomenon and summation of flashes can be demonstrated. Firefly preparations were made by taking either a whole animal whose anterior ventral nerve cord had been severed or an abdominal region separated from the rest of the body, and placing two stainless steel insect pins in the abdominal segments for use as stimulating electrodes. The light organs of glow-worms have usually been dissected out and placed in Ringer's solution held in a lucite chamber, covered so as to form a moist chamber. An electrode was placed on each side of the organ about 2 cm apart. At times glow-worm preparations similar to those of fireflies were studied. The flashes which result from stimulation were recorded by the photomultiplier-amplifier-cathode-ray oscillograph-camera combination described in the previous work (Chang, '54). The

stimulus was a square wave electrical shock whose voltage, duration and frequency could be easily regulated.

Figure 1A shows a series of flash responses of the firefly, *Photuris pennsylvanica,* to repeated stimulation at the rate of 10 per second. Each successive flash has a peak light intensity greater than that which precedes it, and yet, the flashes are well separated. Such stair-case phenomena can be obtained regularly if the central nervous control of the light organ is completely removed. Isolated glow-worm light organs also give off flash responses exhibiting gradual and successive increase of peak intensities to repeated identical stimuli (fig. 1B). The time course of these glow-worm responses are slow, as can be seen in the figure. The stimulation frequency in this case was one every two seconds.

Summation of response, which is so characteristic of muscle, can also be obtained regularly both in firefly and glow-worm preparations as a result of repeated stimulation of faster rates. A record of such responses is shown in figure 1C, where an isolated glow-worm light organ was stimulated at the rate of one per second. Similar summation flashes may also be obtained at will from the firefly light organ.

Although the histology of the photogenic organs of Mnemiopsis has not been thoroughly worked out and the course of muscle fibers near it not certain, the present observations on lampyrids demonstrate definitely that photogenic organs without any associated muscle fibers do possess response characteristics which are quite analogous to those of muscles. Treppe and summation of responses appear to be characteristics of the response of luminous cells to stimulation.

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